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Estradiol up-regulates the stimulatory GTP-binding protein expression in the MCF-7 human mammary carcinoma cell line

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The effect of estradiol treatment of the human mammary carcinoma cell MCF-7 on the adenylyl cyclase system was examined. Treatment with 10 nM estradiol for 72 h increased the basal level of cAMP, and isoproterenol-, PGE_2- or calcitonin-stimulated cAMP production. Estradiol also increased the response to cholera toxin but did not alter the response to forskolin. No significant change in growth rate was observed during the 72 h of estradiol treatment. In MCF-7 cell membranes the responsiveness to isoproterenol, PGE_2, or cholera toxin was also enhanced by estradiol treatment. The cholera toxin-catalyzed ADP-ribosylation of Gsα in MCF-7 cell membranes was significantly increased by 72 h of treatment with estradiol. Consistent with this observation, the level of Gsα immunoreactivity was increased in the estradiol-treated cell membranes. On the other hand, pertussis toxin did not change the responsiveness to isoproterenol, PGE_2 or calcitonin in either control or estradiol-treated cells. In addition, ADP-ribosylation with pertussis toxin also did not reveal any change in Gi. These results clearly indicate that Gs expression is under the control of estradiol, and that this effect may contribute to the increased sensitivity of hormone-stimulated adenylyl cyclase activities in MCF-7 cells.

Estradiol; Gs protein; cAMP; Mammary carcinoma

1. INTRODUCTION

Estrogen has been reported to have an ability to regulate target tissue sensitivity to other extracellular signals by altering the expression and/or function of receptors and guanine nucleotide binding proteins (G proteins). Estradiol treatment increases the number of α-adrenergic receptors in rabbit myometrium [1], while it decreases that of luteinizing hormone receptors in the rabbit corpora lutea [2]. Expression of certain types of G proteins fluctuates in the rat anterior pituitary during the estrous cycle [3], and in the myometrium during gestation [4]. Moreover, estradiol has been shown to enhance pertussis toxin-catalyzed ADP-ribosylation of α-subunits of G proteins in rat striatal neurons [5].

Estrogens play an important role in the growth of both normal and malignant mammary epithelial cells [6,7]. cAMP is another growth modulator for them [8–10]. cAMP levels are elevated in several breast carcinomas [11]. Not only cAMP levels but also β-adrenergic receptor concentration increase in parallel with the growth of mammary glands during pregnancy [12,13]. These observations suggest that estrogens may act as a modulator of the signal transduction pathway, involving cAMP as a second messenger, to stimulate cell growth. However, no precise role of estrogens on hormone-mediated cAMP production in mammary epithelia is known. So we asked whether estradiol modulates the signal transduction system in MCF-7, a hormone-sensitive human mammary carcinoma cell line. We found that, not only the basal level of cAMP but also cAMP production in response to isoproterenol, PGE_2 or calcitonin, was increased after estradiol treatment. In an attempt to better understand this effect of estradiol, we examined the possible modulation of G protein levels by estradiol in MCF-7 cells and found that estradiol induced a significant increase in Gsα protein levels without any alteration in the Giα protein level.

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Abbreviations: PGE_2, prostaglandin E_2; FBS, fetal bovine serum; BSA, bovine serum albumin; IBMX, 3-isobutyl-1-methylxanthine; DTT, dithiothreitol; SDS, sodium dodecylsulfate; GPP, guanosine 5′-imidodiphosphate; EGF, epidermal growth factor; FGF, fibroblast growth factor; MSA, multiplication stimulating activity.

2. MATERIALS AND METHODS

2.1. *Cell culture*

The human mammary carcinoma cell line, MCF-7, was maintained in RPMI 1640 (Flow Laboratories, McLean, VA) supplemented with 10% FBS. For experiments involving estrogen induction, the cells were passaged for at least 1 week in Phenol red-free RPMI 1640 containing 10% heat-inactivated FBS prior to use.

2.2. *Membrane preparation*

After a 72 h treatment with estradiol, plasma membranes were obtained according to the method previously described by Gerwins et

al. [14]. Purified membranes were suspended in 25 mM Tris-HCl (pH 7.5) and stored in aliquots at −80°C until use.

2.3. *Determination of cAMP levels and adenylyl cyclase activity*

MCF-7 cells were plated at a density of 1×10^4/well into 24-well multidishes with 1 ml Phenol red-free RPMI 1640 containing 10% heat-inactivated FBS. After 48 h incubation, 10 nM estradiol or vehicle (0.001% ethanol) was added to culture medium to the desired concentration and the cells were further incubated for 12–72 h. To examine the response to isoproterenol, PGE_2, calcitonin, cholera toxin or forskolin, the medium was replaced with FBS-free medium containing 0.1% BSA and 2 mM IBMX, and test solution was added 30 min thereafter. At the end of a 10 min treatment, cellular cAMP was extracted by sonicating for 10 s in 0.1 N HCl. Adenylyl cyclase activity of the membrane was assayed as described previously [15]. cAMP was measured with a Yamasa RIA kit.

2.4. *ADP-ribosylation of the membrane with cholera toxin*

Cholera toxin (1 mg/100 μl) was preactivated for 15 min at 37°C by treatment with 20 mM DTT in 50 mM K_2HPO_4/KH_2PO_4 (pH 7.0). The incubation mixture (100 μl) contained 100 μg preactivated cholera toxin, 35 or 70 μg membrane protein, 200 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 1 mM ATP, 10 mM thymidine, 5 mM $MgCl_2$, 1 mM GTP, 20 μCi [^{32}P]NAD (Du Pont-NEN Research Products, MA) and 5 μM NAD. After 10 min incubation at 37°C, the reaction was terminated by addition of 1 ml stop solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM NAD. The membrane was collected by centrifugation at 12,000 × *g* for 15 min and washed twice with the stop solution. The membrane pellets were resuspended in 20 μl Laemmli's sample buffer and boiled for 10 min. Samples were subjected to SDS-PAGE according to the method of Laemmli, with 10% acrylamide gels [16]. After completion of electrophoresis, gels were dried and exposed to X-ray films.

2.5. *Immunoblotting of G proteins*

Membranes were solubilized in Laemmli's sample buffer and the proteins were separated using SDS-PAGE as described above. After transfer of proteins to nitrocellulose sheets, immunoreaction was performed as described [17]. The antiserum RM/1 (Du Pont-NEN Research Products, MA), which was generated against the C-terminus of Gsα, was used to detect the 45 kDa and 52 kDa forms of Gsα.

Synthetic salmon calcitonin(1-32) (calcitonin) was kindly donated from Yamanouchi Pharmaceutical Co. (Tokyo, Japan). Statistic analyses were performed using Student's *t*-test.

3. RESULTS

We first examined the effect of estradiol treatment of MCF-7 cells on the responsiveness to isoproterenol, PGE_2 or calcitonin. As indicated previously, MCF-7 cells possess isoproterenol, PGE_2 and calcitonin receptors coupled to adenylyl cyclase [18]. Treatment of 10 nM estradiol for 72 h resulted in an increase in the cAMP production stimulated by these agonists (Fig. 1). In addition, there is a significant increase of basal cAMP levels in MCF-7 cells treated with estradiol as compared with the control (12.1 ± 1.1 pmol/well vs. 7.8 ± 0.5 pmol/well; $P < 0.01$). Time–course studies of the effects of estradiol on calcitonin and PGE_2 responses revealed that they required at least 48 h of

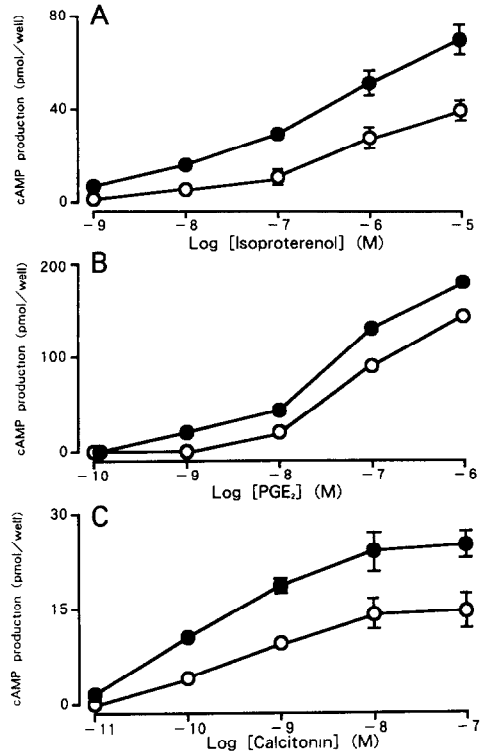

Fig. 1. Effect of estradiol on the dose–response curve for isoproterenol-, PGE_2- or calcitonin-stimulated cAMP production in MCF-7 cells. Cells were preincubated without (○) or with 10 nM estradiol (●) for 72 h. After preincubation, cells were treated with the indicated concentrations of (A) isoproterenol, (B) PGE_2, or (C) calcitonin, for 10 min in the presence of 2 mM IBMX. Each point shows the mean ± S.D. of triplicate determinations from one of two independent experiments.

Table I

Effect of estradiol on cholera toxin- or forskolin-stimulated cAMP production in MCF-7 cells

	Cholera toxin (μg/ml)			Forskolin (nM)		
	0	0.1	1	0	10	100
Control	8.6 ± 0.9	78 ± 6	423 ± 28	7.9 ± 0.4	59 ± 1	318 ± 22
Estradiol	12.7 ± 0.4*	98 ± 7*	561 ± 12*	11.3 ± 1.1*	61 ± 6	290 ± 12

Cells were preincubated with or without 10 nM estradiol for 72 h. After preincubation, cells were treated with the indicated concentrations of cholera toxin for 60 min, or forskolin for 10 min, in the presence of 2 mM IBMX. cAMP production is expressed as pmol/well. Each value represents the mean ± S.D. of triplicate determinations. *$P < 0.01$ compared to the control value.

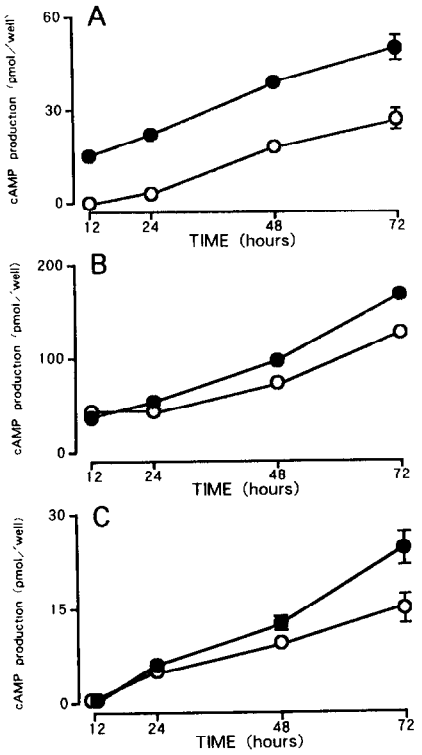

Fig. 2. Time dependence of estradiol effect on isoproterenol-, PGE_2-, or calcitonin-stimulated cAMP production in MCF-7 cells. Cells were preincubated without (○) or with 10 nM estradiol (●) for up to 72 h. After preincubation, cells were treated with (A) 1 μM isoproterenol, (B) 1 μM PGE_2, or (C) 10 nM calcitonin for 10 min in the presence of 2 mM IBMX. Each point shows the mean ± S.D. of triplicate determinations from one of two independent experiments.

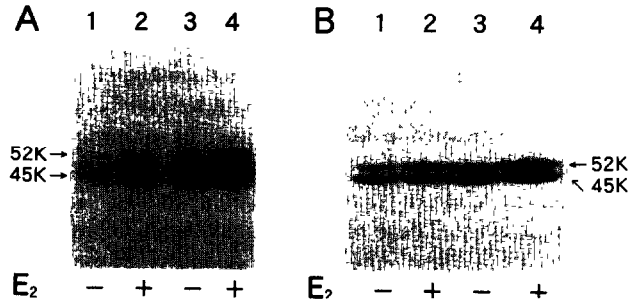

Fig. 3. Quantitation of G proteins in membranes from MCF-7 cells, using ADP-ribosylation and immunoblotting. Membranes were prepared from control cells and cells treated with 10 nM estradiol (E_2) for 72 h. (A) ADP-ribosylation of G proteins with cholera toxin. (B) Immunoblotting of the Gsα subunit. Lanes 1 and 2 were loaded with 35 μg of membrane protein, and lanes 3 and 4 with 70 μg of membrane protein.

estradiol treatment to be evident, while the isoproterenol response was significantly increased after 12 h treatment with estradiol (Fig. 2). No significant change in growth rate was observed during the 72 h of estradiol treatment (data not shown).

Estradiol treatment of MCF-7 cells also increased cholera toxin-stimulated cAMP production, but did not alter forskolin-stimulated cAMP production (Table I). These estradiol effects on the adenylyl cyclase system were also examined using MCF-7 cell membrane preparations (Table II). Although calcitonin-stimulated cAMP production in the cell membrane was too small to be detected, the responsiveness to isoproterenol, PGE_2 or cholera toxin was further enhanced by a 72 h treatment of estradiol.

In order to investigate whether the observed changes in responsiveness to these agonists were due to quantitative or qualitative changes in G proteins, we performed ADP-ribosylation with cholera toxin and immunoblotting with an antibody against the Gsα subunit. Cholera toxin induced the ADP-ribosylation of the 45-kDa and 52-kDa forms of Gsα on SDS-polyacrylamide gels (Fig. 3). The cholera toxin-catalyzed ADP-ribosylation of Gsα of an MCF-7 cell membrane fraction was significantly increased by a 72 h treatment of estradiol, suggesting an increased amount of Gsα. This was confirmed by measuring the level of Gsα by the immunoblotting method.

Pretreatment of cells with pertussis toxin did not alter the responsiveness of cells to isoproterenol, PGE_2 or calcitonin in either control or estradiol-treated cells (data not shown). In addition, ADP-ribosylation with pertussis toxin also did not reveal any change in the level of Gi after 72 h of treatment of estradiol (data not shown).

Table II

Effect of estradiol on isoproterenol-, PGE_2-, or cholera toxin-stimulated cAMP production in MCF-7 cell membranes

	None	Isoproterenol (0.1 mM)	PGE_2 (0.1 mM)	Cholera toxin (20 μg/ml)
Control	2.99 ± 0.15	3.78 ± 0.14	5.02 ± 0.25	4.11 ± 0.13
Estradiol	2.68 ± 0.21	4.54 ± 0.43*	5.89 ± 0.16*	4.65 ± 0.25*

Membranes were prepared from control cells and cells treated with 10 nM estradiol for 72 h. Membranes were treated with the indicated concentrations of isoproterenol, PGE_2 or cholera toxin, for 30 min in the presence of 2 mM IBMX. Cholera toxin was preactivated as described in section 2. cAMP production is expressed as pmol/30 min/μg protein. Each value represents the mean ± S.D. of triplicate determinations. *$P < 0.01$ compared to the control value.

4. DISCUSSION

In the present study, we have demonstrated the enhancement of hormone-stimulated adenylyl cyclase activities by estradiol in MCF-7, a human mammary carcinoma cell line. Associated with this were increases in the specific cholera toxin substrates and in Gsα immunoreactivity. These results suggest that estradiol exerts its stimulating influence on adenylyl cyclase-activating pathway through increasing Gsα expression. On the other hand, inhibitory G protein (Gi) seemed unlikely to be affected by 72 h treatment with estradiol.

There are few reports that describe the regulatory role of estrogens on Gs protein function and/or expression [2–4,19]. Treatment of rats with estradiol induced a significant reduction in immunoreactivity towards most of the pituitary G proteins except Giα1, which remained unchanged, and Gsα42, which increased [3]. Estradiol caused reduction of Gs function in rat luteal cells using reconstitution assay that employs a cyc^- variant of the S49 mouse lymphoma cell line [2]. In rabbit myometrium, while estradiol reduced cholera toxin-catalyzed ADP-ribosylation, the hormone did not alter Gs activity stimulated by GTP, GPP or NaF [19]. These observations have not made it clear whether estrogen-induced modification of G protein function was actually accompanied by altered levels of G proteins. There are several findings that differences in the extent of ADP-ribosylation reflect those in properties of G proteins rather than their total amounts [20,21]. For example, Maus [5] reported that pretreatment of striatal neurons from mouse embryos in primary culture with estradiol enhanced functions of Gi and Go by stabilizing association between heterotrimeric subunits with no change in their total amount. In the present study, we found that estradiol induced similar increases in 45 kDa and 52 kDa Gsα by means of an ADP-ribosylation procedure, along with an increase in the hormone-stimulated adenylyl cyclase activities. Similar increases were also observed in both forms of Gsα by immunoblotting. Therefore, to our knowledge, this is the first report demonstrating that estradiol increased Gsα expression, which resulted in the increased sensitivity of hormone-stimulated adenylyl cyclase activity in MCF-7 cells.

Time–courses of the effects of estradiol on calcitonin and PGE_2 responses were similar, requiring at least 48 h of estradiol treatment to be evident, while the isoproterenol response was significantly increased at 12 h of exposure. Although there has been no report that describes the time–course of estrogen-induced alteration in G protein expression, an early effect of estradiol on isoproterenol-stimulated cAMP production seems unlikely to be mediated by alteration in the Gsα level. Rather, involvement of alteration in β-adrenergic receptor number should be considered, because there are some indications that β-adrenergic receptor number fluctuates within a short time range: (i) increase in β-adrenergic receptor number induced by treatment of osteosarcoma ROS 17/2.8 cells with dexamethasone was observed at 12 h [22], although there have been no such findings with estrogen; (ii) increase in β-adrenergic receptor number in rat mammary gland correlated with parallel increases in plasma estradiol and progesterone levels during pregnancy [12]. It was reported that Gsα interacts with ion channels [23] in addition to adenylyl cyclase. This observation suggests that estradiol might modify other transduction pathways. Further characterization of estradiol regulation of signal transduction pathways could provide insight into other such systems.

The finding that Gsα expression is up-regulated by estradiol in estrogen receptor-positive mammary carcinoma cells is interesting when considering the effect of estrogen on their growth. Estradiol is a potent growth promoter and its effects are, in part, mediated by up-regulation of EGF/TGF-α receptor number [24] and secretion of growth factors such as TGF-α [25] and IGF-I [26]. However, in serum-free medium, it showed no growth effect on cells from normal, benign, and malignant human mammary cells in primary culture [26], and estradiol was capable of enhancing the growth-stimulating effect of EGF in the same primary culture of human mammary carcinoma. Moreover, in the presence of serum, estradiol could stimulate growth of cells from normal and malignant mammary tissues [27]. These observations suggest that estrogen might act as a modulator of cellular signal transduction pathways of growth factors, rather than acting directly as a mitogen. cAMP also modulates the growth of breast epithelial cells. Although it is not clear whether cAMP alone stimulates or inhibits their growth, possible involvement of the nucleotide in cell growth is suggested from the finding that growth factors (EGF, FGF, or MSA) and cholera toxin have shown a synergistic effect on growth of cells from normal mouse mammary glands and some kinds of mouse mammary tumors [28]. cAMP levels increased in parallel with mammary gland growth during pregnancy [13], and they were elevated in several mammary carcinomas [11]. In addition to up-regulation of EGF/TGF-α receptor number and their secretion, these findings suggest that estrogens might be involved in the growth of normal and malignant mammary tissues by enhancing Gs function and/or expression.

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