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Polyamines Modulate Growth Factor-Induced Membrane Protein Phosphorylation in MCF-7 Human Breast Cancer Cells

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Abstract

Breast cancer cell lines display a wide variety of growth factor receptors, and considerable evidences implicate the importance of signalings from those receptors. A useful prognostic indicator would be the level of activity of a second messenger protein used in common by these receptors. Our studies were designed to obtain preliminary information on the possible role of polyamine as a mediator of the membrane-associated protein phosphorylation and as a regulator of second messenger in mitogenic signal of estrogen or growth factors in MCF-7 human breast cancer cells. DFMO significantly inhibited the phosphorylation induced by E_2, TGF-α and EGF in membrane-associated proteins (154, 134, 116, and 104 kDa). Exogenous polyamines abolished the inhibitory effect of DFMO. Tyrosine phosphorylations of membrane-associated proteins were not increased by E_2 or growth factor treatments and not affected DFMO treatment. Polyamine administration markedly enhanced the tyrosine phosphorylation of membrane-associated proteins (154, 134, and 116 kDa). In the present study, E_2 and TGF-α and EGF enhanced protein phosphorylation in the almost same levels. These data indicate that E_2 and growth factor signaling pathway may cross-talk through various protein kinase which phosphorylated many substrate proteins (154, 134, 116 and 104 kDa). Polyamines may be involved in growth signaling pathway of E_2 and TGF-α or EGF for the cross-talk through regulation of the protein phosphorylation such as 154, 134, 116 and 104 kDa. Polyamine may also selectively interfere with several different protein kinases, and the specific steps in signal transduction system were effected by polyamines.

Key words – E_2, EGF, Membrane, Polyamines, TGF-α

Introduction

Breast cancer cell lines display a wide variety of growth factor receptors, and considerable evidences implicate the importance of signalings from those receptors. A useful prognostic indicator would be the level of activity of a second messenger protein used in common by these receptors.

Estrogen stimulates the proliferation of human breast cancer cells in autocrine/paracrine fashion, by inducing the synthesis and secretion of growth factors; epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), insulin-like growth factor Ⅰ (IGF-Ⅰ), and plate-derived growth factor (PDGF). Human breast cancer cell proliferation is regulated by growth factors that bind to the receptors with intrinsic tyrosine kinase activity including the EGFR [21]. Protein tyrosine kinase activity of the cytosolic and membrane fractions of breast cancer cell is significantly higher compared to the benign or the normal breast tissue [19]. The most well-characterized of these are the receptor tyrosine kinase *erb*B-2 and the EGFR. The c-*erb*B-2 protooncogene (also called *neu* or HER2) encodes a transmembrane tyrosine kinase ($p185^{erbB-2}$)

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that is structurally related to the EGF receptor.

Polyamine is ubiquitous in all prokaryotes and eukaryotes, and is involved in multiple functions required for cell growth, differentiation and transformation. Polyamine is a mediator in many tissues in response to various stimuli such as hormones and growth factors. Cell proliferation and higher transformation induced by growth factor are characterized by increased polyamine biosynthesis and enhanced uptake of polyamines [12]. Alpha-difluoromethlornithine (DFMO) is a highly specific and irreversible inhibitor of ornithine decarboxylase (ODC) which is the first and rate limiting enzyme in polyamine biosynthesis [20]. DFMO inhibits ODC by binding to its active site, thereby preventing polyamine synthesis and cell proliferation. Since its administration causes a significant suppression of cell growth and tumor formation *in vivo* as well as *in vitro*, DFMO is being developed as a chemopreventive agent.

Polyamines may play an important role of multiple function in protein phosphorylation. Changes of second messenger systems such as tyrosine kinase, PKA, and PKC are accompanied with the transition from the normal to the malignant state. Polyamines are also involved in many actions of EGF [15]. In L6 cells and fetal bovine myoblasts, EGF and TGF-α, also a ligand of EGFR, stimulate polyamine biosynthesis, suggesting that the biosynthesis of polyamines is important in the early events induced by EGF [2]. Polyamine functions in signal transduction processes by regulating the activities of phospholipase C [13], protein kinase C [3], and phosphorylation of casein [16] and other proteins. Also, polyamines are involved in retinoblastoma protein phosphorylation, which is necessary for G1/S transition [18]. Even though, the specific steps in the signal transduction affected by polyamines still remain to be largely established.

Previous studies indicate that the polyamine pathway may be deeply interrelated with the autocrine/paracrine control of breast cancer cell proliferation [7]. Polyamine act as mediator of estradiol-stimulated growth of several human breast cancer cell lines [13,6]. Activation of the polyamine pathway by promoting several key steps involved in proliferation causes the transition from a hormone-dependent to a hormone-independent breast cancer phenotype [14].

It was reported that ER function may be regulated by estrogenic ligands as well as by cross-talking membrane receptors for growth factors through phosphorylation of the human ER. Recently, it was suggested that signal transduction by the growth factor and E_2 may be modulated by polyamine, but the exact mechanisms for this cross-talk are pooly understood. Membrane-associated tyrosine kinase is important in growth signal transduction of human breast cancer cells. The identification of specific phosphoprotein targets is also important to understand the mechanisms that control the cell growth in cancer.

Our experiments were designed to obtain preliminary information on the possible role of polyamine as a mediator of the membrane-associated protein phosphorylation and as a regulator of second messenger in mitogenic signal of estrogen or growth factors in MCF-7 human breast cancer cells.

Materials and Methods

Chemicals

TGF-α, EGF, 17β-estradiol, putrescine (tetramethylenediamine), spermidine (N-[3-aminopropyl]-1,4-butanediamine), spermine (N, N' bis-[3-aminopropyl]-1, 4-butanediamine) were purchased from Sigma Chemical Co. [γ-^{32}P]ATP (>5000 mCi/mmol) was obtained from Amersham Korea Ltd. α-Difluoromethylornithine (DFMO) was obtained from Dr. Levenson ILEX Oncology Inc. (San Antonio, Texas, USA). Anti-phosphotyrosine antibody 4G10 (mouse monoclonal IgG) were purchased from Upstate Biotechnology. All other reagents. were of the highest grade commercially available.

Cell line and culture condition

MCF-7 cells obtained from the Korean Cell Line Bank

were grown in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and 1,000 mg/L glucose containing 10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Cells were maintained at 37℃ in a humidified atmosphere containing 5% CO_2/95% air. Medium was changed every 2 or 3 days. Cell number was determined by Coulter Counter (Model Z1, Coulter Electronics Ltd. USA). For all experiments, cells were grown for 72 hr in medium without phenol red prior to any treatment in order to avoid the estrogenic effect of phenol red [1].

Membrane preparation and phosphorylation

Cell membranes were isolated by using a modified procedure described by Mueller *et al.* [17]. Cells were inoculated at a density of 2×10^5 cells/ml into 10 mm tissue culture dishes in phenol red free DMEM. All procedures were performed under 4 ℃ unless mentioned specifically After 72 hr incubation, sub-confluent cultures (70~80%) were washed twice with 50 mM tris-HCl (pH7.4), and scrapped off with a rubber policeman, then sonicated for 5 sec in lysis buffer containing 50 mM tris-HCl (pH 7.4), 5 mM EDTA, 5 mM DTT, 2 mM PMSF, and 125 μM leupeptin. All debris and nuclei were removed by centrifugation at 600 ×g for 8 min. The supernatant was loaded on a sucrose cushion (35% sucrose in NaCl/Pi) and further centrifuged at 240,000 ×g for 30 min. The interface was collected, diluted in 10 mM Hepes, pH 7.5, and centrifuged at 240,000 ×g for 30 min. The resulting pellet was resuspended in kinase buffer containing 10 mM $MgCl_2$,and 20 μM ATP, then 50 μl membrane aliquots were incubated for 5 min at 4℃ with the experimental treatments. The reaction mixtures were incubated for 1 min with 5 μCi [γ-^{32}P] ATP and the reaction was stopped by the addition of SDS-PAGE sample buffer.

Radioimmunoprecipitation of tyrosine phosphorylated protein

Radioimmunoprecipitation assay (RIPA) was performed by the method of Hartley *et al.* [8] with some modifications. For each reaction, ^{32}P-labeled lysates were diluted in RIPA buffer with 1 mM PMSF and 10 μg/ml aprotinin to obtain a final volume of 200 μl. The above sample was reacted with 20 μl of monoclonal anti-phosphotyrosine antibody (1:500) overnight at 4°C with gentle rotation. Immune complexes were precipitated with 200 μl of pre-swelled Protein A Sepharose (0.05 g protein A/ml RIPA buffer with 1 mg BSA) for additional 1.5 hr at 4°C. Bound immune complexes were washed 4 times with 1.0 ml RIPA lysing buffer. The immune complexes were dissociated from the beads by boiling in 50 μl SDS-PAGE sample buffer for 3 min. Supernatant was separated by micro centrifugation for 2 min.

SDS-Polyacrylamide gel electrophoresis and fluorescence image analyzer system

Phosphorylated proteins were subjected to SDS-PAGE on 10% acrylamide slab gel using the method of Laemmli [11]. Dried, gels were exposed to the Image Plate, and scanned using the Image Reader program of FLA-2000 (Fuji Photo Co., Japan). The signal intensities were quantified with the Image Gauge 3.1.1 of FLA-2000.

Statistical analysis

All experiments were carried out at least three times. Data are presented as mean±standard deviation (SD). Statistical significance of difference between untreated control and treated groups in protein phosphorylation was determined using one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test. In all cases, a p value less than 0.05 was considered significant.

Results

Inhibition of E_2-, TGF-α-, or EGF-induced total protein phosphorylation by DFMO in the cell membrane-associated proteins

Principally, high molecular weight proteins of 154, 134,

116, and 104 kDa were detected in the membrane preparation. As shown in Fig. 1A and Table 1, phosphorylation of the most proteins were enhanced by the treatment of E_2, TGF-α, or EGF. The maximum stimulatory effect in phosphorylation was found at 134 kDa protein, which increased the phosphorylation to 190% of the untreated control in TGF-α-induced membrane-associated proteins. But, the phosphorylation was inhibited to 56% of the untreated control and to 29% of TGF-α treatment by 5 mM DFMO administration. In E_2, TGF-α, or EGF treatment, the phosphorylation of 116 kDa protein was slightly decreased to 78~94% of control in 5 mM DFMO co-treatment. These decrease were statistically significant compared to E_2-, TGF-α-, or EGF-induced phosphorylation (Table 1). In 104 kDa protein, DFMO administration prevented total phosphorylation to less than that in E_2, TGF-α, or EGF-stimulated cell membrane fraction.

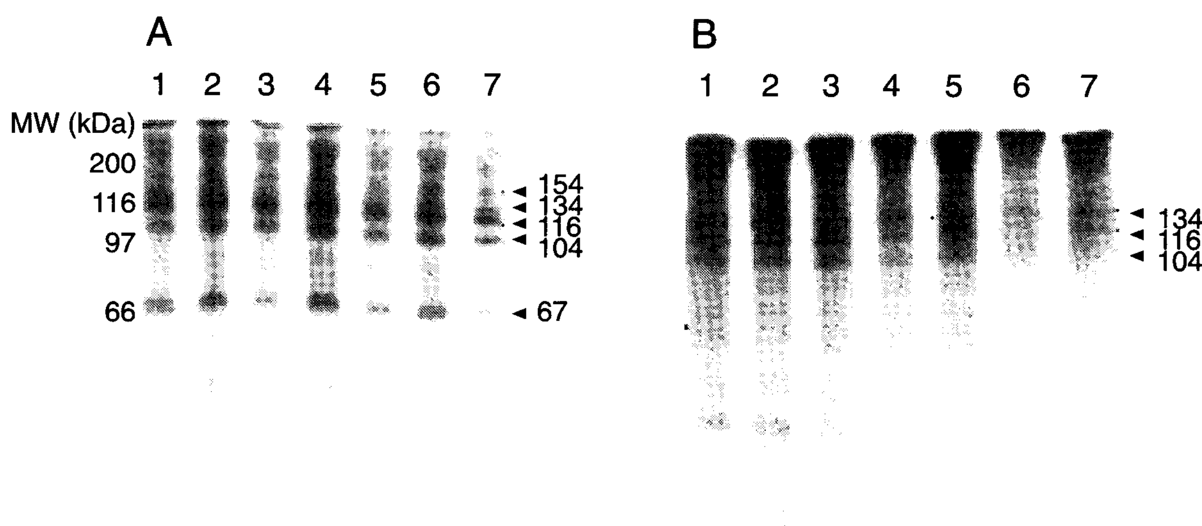


Fig. 1. Effect of DFMO on E_2, TGF-α, or EGF-induced phosphorylation in the cell membrane-associated proteins.
A. autoradiogram of total phosphoprotein. B. autoradiogram of tyrosine phosphoprotein. Indications on the left of each gel are molecular weight standards. Lane 1; untreated control, 2; 10 nM E_2, 3; E_2+5 mM DFMO 4; TGF-α (5 ng/ml), 5; TGF-α+DFMO, 6; EGF(5ng/ml), 7; EGF+ DFMO. An equal amount of the membrane protein was loaded on each lane. The data presented here is representative of at least 3 separate experiments.

Table 1. Inhibitory effect of DFMO on total phosphoprotein induced by E_2, TGF-α or EGF in the cell membrane-associated proteins (% of the control)

M.W.(kDa)	Treatment					
	E_2	E_2+DFMO	TGF-α	TGF-α+DFMO	EGF	EGF+DFMO
154	161 ± 40^{a}	61 ± 21^{ab}	146 ± 9^{a}	52 ± 4^{ac}	142 ± 8^{a}	64 ± 17^{ad}
134	145 ± 4^{a}	58 ± 10^{ab}	190 ± 35^{a}	56 ± 11^{ac}	128 ± 11^{a}	64 ± 5^{ad}
116	146 ± 37^{a}	94 ± 23^{b}	174 ± 15^{a}	78 ± 4^{c}	124 ± 1^{a}	73 ± 1^{ad}
104	125 ± 18^{a}	75 ± 18^{ab}	166 ± 3^{a}	60 ± 6^{ac}	124 ± 4^{a}	66 ± 10^{ad}
67	142 ± 21^{a}	52 ± 25^{ab}	150 ± 1^{a}	58 ± 8^{ac}	137 ± 3^{a}	73 ± 4^{ad}

The data presented here is the mean±SD of at least 3 separate experiments.
$^{a}p<0.05$ vs. control, $^{b}p<0.05$ vs. E_2, $^{c}p<0.05$ vs. TGF-α, $^{d}p<0.05$ vs. EGF

Effects of DFMO, E_2-, TGF-α-, or EGF on tyrosine phosphorylation in the cell membrane-associated proteins

Tyrosine phosphorylations of the membrane proteins were not increased by the treatment of E_2, TGF-α, or EGF, rather it was slightly decreased. Furthermore, DFMO administration slightly increased the tyrosine phosphorylation in three membrane-associated phosphotyrosine proteins. Especially, 134 and 116 kDa proteins were significantly increased by 5 mM DFMO administration in E_2, and TGF-α treatment, respectively (Table 2, Fig. 1B).

Effects of polyamines on protein phosphorylation induced by E_2, TGF-α or EGF

In the cell membrane preparation, polyamines blocked the inhibitory effect of DFMO on protein phosphorylation. Three polyamines exerted similar effect on E_2, TGF-α, or EGF-induced phosphorylation. In the case of 154 kDa proteins, spermidine showed the highest recovery effect in E_2 or TGF-α treated along with DFMO, respectively (Fig 2). While putrescine did not give any noticeable recovery effect in EGF treatment, spermine gave similar reversal effects to spermidine against the inhibitory effect of DFMO (data not shown). Spermine at 1 mM completely overcame the inhibitory effects of DFMO on E_2-induced phosphorylation of 154 kDa protein up to E_2 treatment level (data not shown). Similar results were founded with putrescine in TGF-α treatment (Fig. 3). Three polyamines tested blocked the inhibitory effect of DFMO on phosphorylation of 134 kDa protein in E , TGF-α, and EGF treatment. Putrescine and spermidine did not give any significant effect on 116 kDa protein in E_2, and TGF-α treatment, and spermine did not give effect on protein phoshporylation in TGF-α and EGF treatment. Since the phosphorylation of 116 kDa protein was not much inhibited by DFMO, polyamine did not give any significant effect on protein phosphorylation. All three polyamines could reverse the inhibitory effect of DFMO on EGF-stimulated phosphorylation at 104 kDa protein, but exerted only partial recovery effect on the phosphorylation in E_2, or TGF-α-induction. Among the tested polyamines, spermidine most effectively abolished the inhibition of phosphorylation in 104 protein by DFMO with TGF-α stimulation.

Effects of exogenous polyamines on tyrosine phosphorylation in DFMO-treated cell membrane preparation

Among the four major cell membrane-associated phosphoproteins, three proteins of 134, 116, and 104 kDa were identified as phosphotyrosine proteins by radioimmumoprecipitation (Fig. 1B). Even though the total phosphorylation of these three proteins were increased by E_2, TGF-α, or EGF treatment, as was shown in Table 1, enhancement of tyrosine phosphorylation could not be found in any one of those three proteins. However polyamine administration significantly enhanced the phosphorylation in all three proteins. The maximum enhancing

Table 2. Effect of DFMO, E_2, TGF-α or EGF on tyrosine phosphorylation in the cell membrane-associated proteins (% of the control)

M.W.(kDa)	Treatment					
	E_2	E_2+DFMO	TGF-α	TGF-α+DFMO	EGF	EGF+DFMO
134	102±13	136±38[ab]	89±23	110±18	85±28	109±45
116	83±30	123±6	91± 9	129±40[ac]	84±27	121±62
104	90±12	126±6	74±10[a]	93±20	73± 3	112±11

The data presented here is the mean±SD of at least 3 separate experiments.
[a]$p<0.05$ vs. control, [b]$p<0.05$ vs. E_2, [c]$p<0.05$ vs. TGF-α

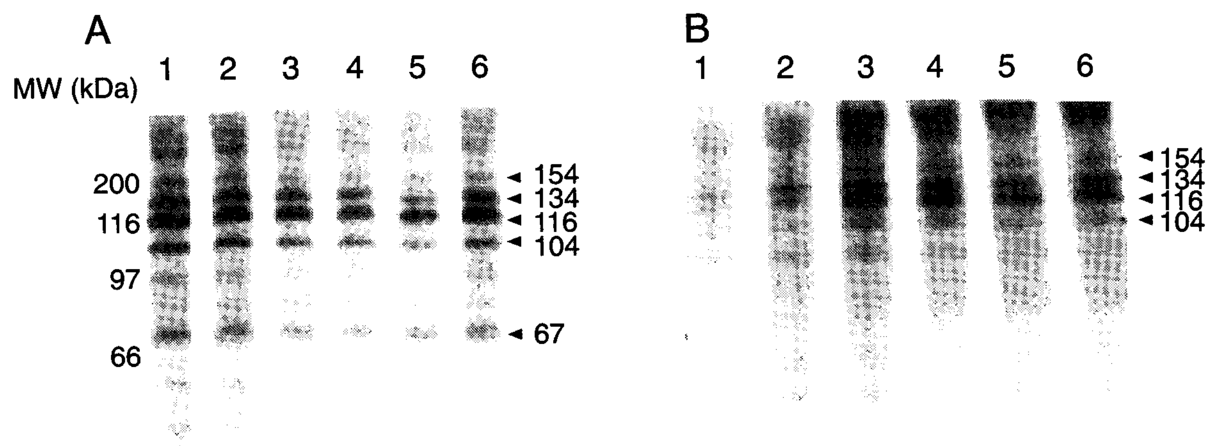
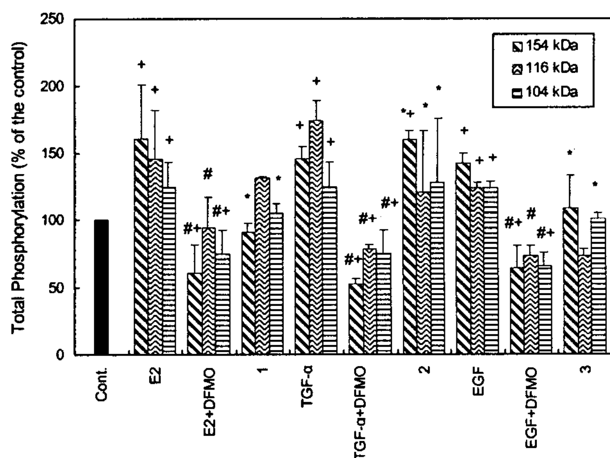


Fig. 2. Polyamine reverses the inhibitory effects of DFMO on E_2-, TGF-α, or EGF-induced total phosphorylation.

1; 1 mM spermidine+10 nM E_2+5 mM DFMO, 2; 1 mM spermidine+TGF-α (5 ng/ml)+DFMO, 3; 1 mM spermidine +EGF (5 ng/ml)+DFMO. The data presented here is the average of at least 3 separate experiments. + $p<0.05$ vs. control; #$p<0.05$ vs. treatment(E_2, TGF-α, or EGF); *$p<0.05$ vs. DFMO.



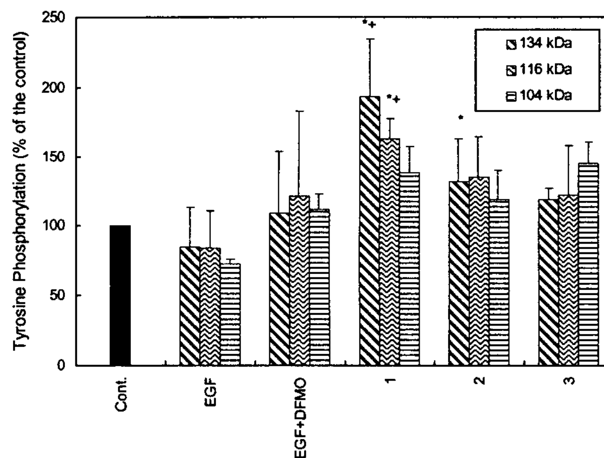


Fig. 3. Effects of exogenous polyamin on DFMO-suppressed protein phosphorylation in the cell membrane-associated proteins.

A. autoradiogram of total phosphoprotein. B. autoradiogram of tyrosine phosphoprotein (see Fig. 1 for markers on each gel). Lane 1; untreated control, 2; TGF-α (5 ng/ml), 3; TGF-α +5 mM DFMO, 4,5,6; TGF-α +DFMO+ 0.1, 0.5, 1.0 mM putrescine, respectively. The data presented here is representative of at least 3 separate experiments.

Fig. 4. Recovery effect of exogenous putrescine on DFMO-suppressed tyrosine phosphorylation.

1,2,3; EGF (5 ng/ml)+DFMO (5 mM)+0.1, 0,5, 1.0 mM putrescine, respectively. The data presented here is the average of at least 3 separate experiments. +$p<0.05$ vs. control; #$p<0.05$ vs. treatment(E_2, TGF-α, or EGF); *$p<0.05$ vs. DFMO.

effect was achieved at 1 mM putrescine, which increased the tyrosine phosphorylation of 116 kDa protein to 300% of untreated control in E_2, treatment (data not shown). But DFMO did not effectively blocked the tyrosine phosphorylation of protein in the membrane preparation. All three polyamines exerted very similar effect on phosphorylation in E_2 and TGF-α treatment. To the contrary, the tyrosine phosphorylation of 116 kDa protein

was slightly increased by 5 mM DFMO in the EGF treatment (Table 2). Enhanced tyrosine phosphorylation by DFMO treatment was strongly accelerated by 0.05 mM putrescine, but the higher concentration of purescine decreased to control level in tyrosine phosphorylation (Fig. 4).

Discussion

Protein phosphorylation is an important regulatory mechanism in response to the action of growth factors and oncogene products. A large number of protein kinases function as signal transducers through phosphorylating tyrosine or serine/threonine residues on critical substrates. Activation of tyrosine kinase at the cell membrane usually correlates with stimulation of cell growth. Although E_2 directly carries out its function through cytosolic and/or nuclear ER, it may also partially stimulate the phosphorylation of many membrane proteins in conjunction with other unknown phosphorylation mechanisms.

Our data showed that phosphorylation was stimulated by E_2, TGF-α, and EGF in membrane-associated proteins. E_2, TGF-α, or EGF treatment induced very similar protein phosphorylation in membrane fraction. Ignar-Trowbridge *et al.* [9] suggested an interaction between EGFR signaling pathway and the ER because EGFR stimulation reproduced many of the effects of estrogen. In the present study, E_2 and TGF-α or EGF induced the same protein phosphorylation. These data indicate that E_2 and growth factor signaling pathway may cross-talk through various protein kinase which phosphorylated many substrate proteins (154, 134, 116 and 104 kDa).

DFMO significantly inhibited the phosphorylation induced by E_2, TGF-α and EGF in membrane-associated proteins (154, 134, 116, and 104 kDa). Also, exogenous polyamines abolished the inhibitory effect of DFMO. Tyrosine phosphorylations of membrane-associated proteins were not increased by E_2 or growth factor treatments, and that of 134 and 116 kDa proteins were slightly increased by DFMO. Polyamine administration did not reversed the effect of DFMO in these proteins, moreover markedly enhanced the tyrosine phosphorylation of membrane-associated proteins (154, 134, and 116 kDa) except putrescine in EGF treatment. If these proteins have many serine/threonine sites than tyrosine sites, increased tyrosine phosphorylation by DFMO might be resulted from the reduction of serine or threonine phosphoryaltion. Otherwise, tyrosine phosphorylation of these protein may be enhanced by DFMO administration. Purescine at 0.05 mM increased the tyrosine phosphorylation in EGF treatment, but 1 mM putrescine decreased that to the control level. The above discrepancy can be explained by the polyamine's multiple effects on protein phosphorylation pathways. Polyamine appeared to increase the protein phosphorylation through the regulation of polyamine-dependent protein kinase. It was showed that polyamine-sensitive protein kinase exerts a multifuntional protein kinase including casein kinase II [22]. Also, previous data indicated that polyamine inhibited the EGF-stimulated EGFR tyrosine kinase activity in A431 human epidermoid carcinoma cells [5]. Polyamines have also been reported to inhibit PKC as well as PKA [4]. Polyamine regulated protein tyrosine phosphorylation in the effect of green tea polyphenols on Ehrlich ascites tumor cells *in vitro* [10]. These data suggested that polyamines may exert at least part of their biological action through an effect upon selective protein phosphorylation systems.

Accumulated evidence suggests that the sum total of growth-factor receptor activity may be a strong determinant of breast cancer malignancy. Activated second messenger protein which is common to all receptors could be an excellent prognostic indicator and, potentially, a therapeutic target. This approach would appear to be a potentially useful one for treating breast cancer in various stage.

Polyamine may selectively interfere with several different protein kinases, and the specific steps in the signal transduction system were effected by polyamines. Poly-

amine binding to the substrate protein may result in a conformational change of protein which, in turn, decreases the accessibility, cation dependency, or protein-protein interactions of proteins. Polyamines may also influence the extent of cell proliferation by modulating the rate of phosphorylation of proteins.

In conclusion, protein phosphorylations induced by E_2 and TGF-α are mediated by multiple phosphorylation pathways in menbrane-associated proteins. Polyamines may be involved in growth signaling pathway of E_2 and TGF-α or EGF for the cross-talk through regulation of the protein phosphorylation such as 154, 134, 116 and 104 kDa. The results from this preliminary work can be used further to elucidate the precise roles of polyamine in the novel cross-talk between estrogen and growth factor signaling pathway.

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초록 : MCF-7 유방암세포에 있어서 growth factor에 의해 유도된 막 단백질의 인산화에 대한 폴리아민의 조절

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유방암세포는 여러 종류의 성장인자 수용체를 가지며, 이를 통해 성장신호를 전달한다. 따라서, 이러한 수용체들의 신호전달 경로에 있는 공통적인 2차전달자들의 조절기작을 밝히는 것이 중요하다. 이 연구는 MCF-7 cell에 있어서, 에스트로젠과 TGF-α, EGF와 같은 성장인자의 mitogenic signal을 전달하는 second messenger에 폴리아민이 어떤 영향을 미치는지, 또 membrane-associated proteins의 인산화에 폴리아민이 어떤 조절기작을 가지는지를 알아보고자 한다. 폴리아민 생합성 억제제인 DFMO는 154, 134, 116, 104 kDa의 membrane-associated proteins의 인산화를 억제하였고, DFMO에 의해 억제된 단백질 인산화는 폴리아민 첨가로 다시 회복되었다. E_2, TGF-α, EGF, DFMO는 모두 단백질의 타이로신 인산화에는 크게 영향을 미치지 않았으나, 처리해준 폴리아민에 의해 154, 134, 116 kDa의 단백질의 타이로신 인산화는 급격히 증가하였다. 또한 E_2, TGF-α, EGF는 모두 같은 단백질에서 유사하게 인산화를 유도하였다. 이러한 결과로 볼 때, E_2와 TGF-α, EGF와 같은 성장촉진인자의 signaling pathway는 154, 134, 116, 104 kDa 단백질을 기질로 하는 여러 가지 다양한 종류의 protein kinase를 통해서 서로 cross-talk하고 있으며, 폴리아민은 154, 134, 116, 104 kDa와 같은 여러 가지 membrane-associated proteins의 인산화를 조절함으로서 이러한 cross-talk pathway에 관여하고 있는 것으로 사려된다.