Polyamine biosynthesis inhibitors alter protein–protein interactions involving estrogen receptor in MCF-7 breast cancer cells

T Thomas[1,3,4], N Shah[1], C M Klinge[5], C A Faaland[2], S Adihkarakunnathu[1], M A Gallo[1,3,4] and **T J Thomas[2,4]**

[1]Department of Environmental and Community Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA
[2]Department of Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA
[3]Environmental and Occupational Health Sciences Institute, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA
[4]The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA
[5]Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, Kentucky 40292, USA

(Requests for offprints should be addressed to T Thomas, Clinical Academic Building, Room 7090, Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA)

ABSTRACT

We investigated the effects of polyamine biosynthesis inhibition on the estrogenic signaling pathway of MCF-7 breast cancer cells using a protein–protein interaction system. Estrogen receptor (ER) linked to glutathione-S-transferase (GST) was used to examine the effects of two polyamine biosynthesis inhibitors, difluoromethylornithine (DFMO) and CGP 48664. ER was specifically associated with a 45 kDa protein in control cells. In cells treated with estradiol, nine proteins were associated with ER. Cells treated with polyamine biosynthesis inhibitors in the absence of estradiol retained the binding of their ER with a 45 kDa protein and the ER also showed low-affinity interactions with a number of cellular proteins; however, these associations were decreased by the presence of estradiol and the inhibitors. When samples from the estradiol+DFMO treatment group were incubated with spermidine prior to GST-ER pull down assay, an increased association of several proteins with ER was detected. The intensity of the ER-associated 45 kDa protein increased by 10-fold in the presence of 1000 μM spermidine. These results indicate a specific role for spermidine in ER association of proteins. Western blot analysis of samples eluted from GST-ER showed the presence of chicken ovalbumin upstream promoter-transcription factor, an orphan nuclear receptor, and the endogenous full-length ER. These results show that multiple proteins associate with ER and that the binding of some of these proteins is highly sensitive to intracellular polyamine concentrations. Overall, our results indicate the importance of the polyamine pathway in the gene regulatory function of estradiol in breast cancer cells.
Journal of Molecular Endocrinology (1999) **22,** 131–139

INTRODUCTION

Polyamines – putrescine, spermidine and spermine – are organic cations with multiple functions in cell proliferation, differentiation and apoptosis (Tabor & Tabor 1984, Heby & Persson 1990, Poulin *et al*. 1993, McCloskey *et al*. 1996, Hu & Pegg 1997). They interact with cellular macromolecules such as DNA, RNA, proteins and phospholipids and alter their structure and conformation. In breast cancer cells, growth stimulatory agents such as estradiol up-regulate the activity of enzymes involved in polyamine biosynthesis pathways, ornithine decarboxylase (ODC) and S-adenosyl

methionine decarboxylase (SAMDC), and increase intracellular polyamine levels (Thomas & Thomas 1993, Thomas *et al*. 1996). An inhibitor of ODC, difluoromethylornithine (DFMO), blocks cell proliferation and this effect can be reversed by exogenous putrescine or spermidine (Metcalf *et al*. 1978, Tabor & Tabor 1984, Thomas & Kiang 1987*a*). An inhibitor of SAMDC, CGP 48664, also inhibits breast cancer cell growth in a reversible manner (Thomas *et al*. 1996). Polyamine-mediated modulation of cell growth and the ability of ODC to facilitate the action of src (Moshier *et al*. 1993) and Ki-ras (Tabib & Bachrach 1994) oncogenes suggest a role for polyamines in neoplastic cell growth. Recent studies on transgenic mice showed that constitutively elevated ODC expression acts as an endogenous tumor promoter in carcinogen-initiated epithelial cells and polyamine depletion by DFMO causes suppression of tumor growth (Smith *et al*. 1998). Signal transduction through the epidermal growth factor receptor and nuclear receptors is also reported to be modulated by polyamines (Manni & Wright 1984, Manni 1989, Faaland *et al*. 1995, Thomas *et al*. 1995), but the mechanism(s) for this crosstalk is unknown.

Steroid and thyroid hormones, as well as lipophilic vitamins, act through a family of nuclear receptors that activate or repress the transcription of responsive genes after their binding to the appropriate ligands (Beato *et al*. 1995, Katzenellenbogen *et al*. 1996). Ligand binding initiates a cascade of intracellular events including the dissociation of heat shock proteins from the nuclear receptors, association of the receptor with other transcription factors, and the binding of the receptor to regulatory sites of the gene to facilitate DNA conformational changes and the action of RNA polymerase. Therefore, the action of the ligand is screened first through the strength of the ligand–receptor interaction, then by protein–protein interactions involving competing transcription factors and auxiliary proteins, and thirdly by the affinity of the ligand–receptor complex for the DNA sites, the hormone response element. A large number of receptor-associated proteins that act as co-regulators (co-activators or co-repressors) of nuclear receptors are being characterized and their functional interactions with gene regulatory proteins are an active area of current investigation (Halachmi *et al*. 1994, Onate *et al*. 1995, Horwitz *et al*. 1996, Lavinsky *et al*. 1998). Often the conserved interaction site of the co-activator is as small as five amino acids and appears to fit into a hydrophobic cleft in the ligand-binding domain of the nuclear receptor (Feng *et al*. 1998).

In the present study, we questioned whether polyamines are involved in gene regulation by modulating the interaction of estrogen receptor (ER) and other proteins. We used a glutathione-S-transferase (GST)-ER fusion protein (consisting of the ligand binding domain of ER) for this purpose. In this assay, cellular proteins that bind to the fusion protein are separated and analyzed by PAGE. We used cellular extracts from MCF-7 cells treated with DFMO or CGP 48664 to examine the effects of polyamine depletion on ER interaction with other proteins. Our results indicate that, in the presence of estradiol, ER association with cellular proteins is repressed by polyamine depletion in a reversible manner.

MATERIALS AND METHODS

Materials

CGP 48664 was obtained from Ciba-Geigy Ltd, Basel, Switzerland. DFMO was from Marrion Merrell Dow, Cincinnati, OH, USA. GST-ER-transformed *Escherichia coli* was obtained from Dr Myles Brown (Harvard Medical School, Boston, MA, USA). The GST-ER plasmid consisted of nucleic acids coding for amino acids 300–596 of ER, comprising the hormone binding domain as well as the nonacidic activation domain (AF-2) cloned downstream to the GST gene (Halachmi *et al*. 1994). [^{35}S]methionine was purchased from New England Nuclear (Boston, MA, USA). Other reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Long Island, NY, USA) with 100 μg/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin, 2 μg/ml insulin, 0·5 mM sodium pyruvate, 10 mM nonessential amino acids, 2 mM L-glutamine and 10% fetal bovine serum (Thomas & Kiang 1987*a*, Thomas *et al*. 1996). Prior to each experiment, cells were grown for 2 weeks in phenol red-free DMEM containing serum treated with dextran-coated charcoal (DCC), to remove serum-derived estrogenic compounds (Thomas & Thomas 1993, Thomas *et al*. 1996). Isoleucine-free DMEM was used for arresting cells in G1 phase. Labeling with [^{35}S]methionine was conducted using methionine-free DMEM.

Proliferation assay

Briefly, 5×10^5 cells were seeded in 60 mm culture dishes in DMEM supplemented with 10% DCC-treated serum and additives. After 16 h of plating, cells were treated with 1 mM DFMO or 1 μM CGP 48664 for 24 h. Cells were then changed to isoleucine-free media for 30 h with appropriate drugs. Cells were then allowed to progress in cell cycle in medium containing isoleucine, 4 nM estradiol and/or the drugs. After 15 h, [^{3}H]thymidine (2 μCi/ml) was added to the medium. After 1 h at 37 °C, the cells were washed with PBS and the radioactive thymidine incorporated in cellular DNA was quantified by liquid scintillation counting (Faaland *et al*. 1995).

Polyamine assay

Cells (2×10^6) were treated in the same manner as for the proliferation assay. They were then harvested and cell pellets treated with 300 μl of 8% sulfosalicylic acid and sonicated. The solution was incubated on ice for 1 h and centrifuged at 10 000 ***g*** for 5 min in a microfuge to remove the precipitated proteins. Intracellular polyamine levels were determined by an HPLC technique using 1,6-diaminohexane.2HCl as internal standard (Thomas & Thomas 1993, Thomas *et al*. 1996).

GST pull down assay

Cells (3×10^6) were plated in T-75 flasks and treated with the drugs as described under the proliferation assay. Ten flasks were used for each treatment group. We added [^{35}S]methionine (0·1 mCi/ml) 12 h after initiation of the cell cycle, in methionine-free DMEM. Cells were harvested 4 h after the addition of [^{35}S]methionine. Cellular lysate was prepared in 1·5 ml of a buffer containing 50 mM Tris–HCl (pH 7·4), 5 mM EDTA, 150 mM NaCl, 0·5% NP-40, 1 mM phenyl methylsulfonyl fluoride, 1 mg/ml leupeptin and 1 mg/ml aprotinin (Halachmi *et al*. 1994). *E. coli* with GST-ER was seeded in Luria broth medium and allowed to grow until the absorbance value reached 0·6 at 600 nm. GST-ER fusion protein was induced by the addition of isopropyl β-D-thiogalactoside. After 3 h of growth, bacterial cells were collected and lysed in a buffer containing 0·5% NP-40, 1 mM EDTA, 20 mM Tris pH 8 and 100 mM NaCl (NETN). After sonication and centrifugation, the supernatant was incubated with 250 μl aliquots of glutathione–Sepharose beads. The Sepharose beads were washed extensively with NETN buffer and then incubated with 100 μl of cellular extract at 4 °C for 16 h. After three washes with NETN buffer, bound proteins were eluted with 100 μl of 20 mM glutathione. Proteins were then analyzed by 12% denaturing polyacrylamide gels and signals were amplified by fluorography.

In order to examine the effect of exogenous spermidine on ER association of proteins, aliquots of cell lysate from the estradiol+DFMO group were incubated with different concentrations of spermidine for 90 min at 4 °C. Samples were then incubated with GST-ER-loaded glutathione–Sepharose beads and subsequently eluted with 20 mM glutathione.

Western blot analysis

Proteins from GST-ER pull down experiments were electrophoretically separated on a 10% polyacrylamide gel. Pre-stained molecular mass markers (Sigma Chemical Co., SDS-7B) were loaded in one of the lanes. The proteins were transferred to PVDF Immobilon membrane (Millipore, Bedford, MA, USA). Membranes were incubated in 8% BSA in Tris-buffered saline (TBS, pH 7·6) containing 0·1% Tween overnight to saturate the nonspecific binding sites. The membranes were incubated either with a 1:1000 dilution of anti-ERα (AER 320, Lab Vision Corp., Fremont, CA, USA) or with a 1:5000 dilution of polyclonal anti-chicken ovalbumin upstream promoter-transcription factor (COUP-TF) antiserum (generously provided by Janet E Mertz of the University of Wisconsin, Madison, WI, USA) in 8% BSA–TBS–Tween buffer for 1 h at room temperature. The membranes were washed three times with a large volume of TBS–Tween for a total of 30 min. They were then incubated with a 1:3000 or 1:10 000 dilution of secondary antibody in 8% BSA–TBS–Tween (Klinge *et al*. 1997). Proteins were visualized with a chemiluminescence-based detection system. A series of exposures ranging from 5 to 30 s time periods was conducted. Identity of protein bands was confirmed using positive control samples. Intensities of the signals were quantified using a Bio-Rad laser densitometric scanner (Bio-Rad Labs, Richmond, CA, USA). Protein levels were verified by Ponceau dye staining of the blot.

RESULTS

Cell growth inhibitory concentrations of DFMO and CGP 48664 were chosen from our previous dose-dependence experiments (Thomas & Kiang 1987*a*, Thomas & Thomas 1993, Thomas *et al*. 1996). One millimolar DFMO and 1 μM CGP 48664 were found to be growth inhibitory, but not

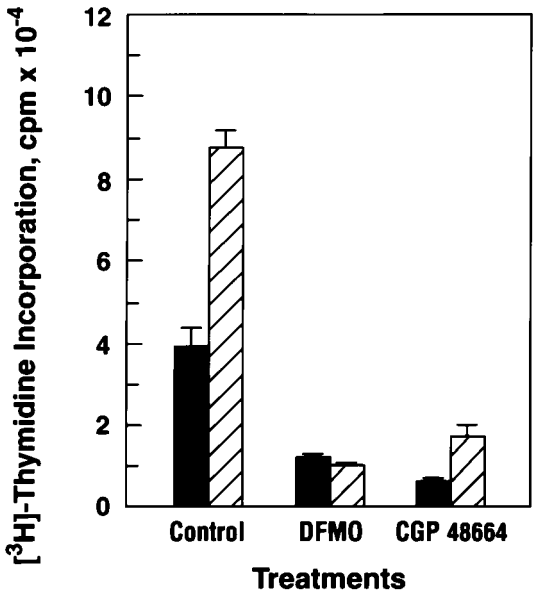

FIGURE 1. Effect of DFMO and CGP 48664 on [^{3}H]thymidine incorporation in the presence (hatched bars) or absence (solid bars) of 4 nM estradiol. G1-synchronized cells progressing in cell cycle for 15 h were used for the determination of DNA synthesis for 1 h. Data represent means and standard deviations from two triplicate experiments.

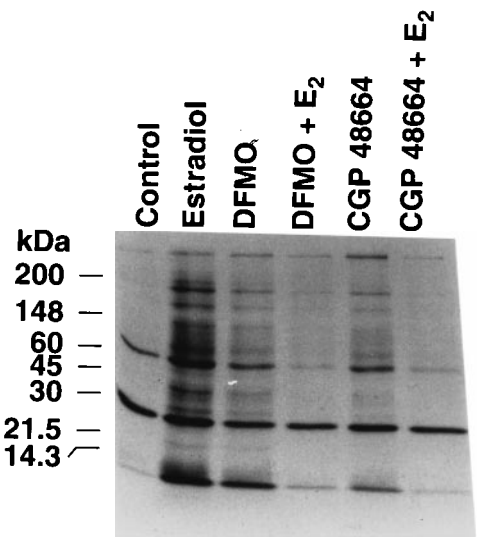

FIGURE 2. Effect of estradiol, DFMO and CGP 48664 on ER-associated proteins in MCF-7 cells. G1-synchronized cells progressing in cell cycle were labeled for 4 h with [^{35}S]methionine. Proteins were detected by PAGE and fluorography. Positions of molecular mass markers were indicated from the mobilities of multicolor markers (Novex, San Diego, CA, USA) and ^{14}C-labeled proteins (Amersham, Arlington Heights, IL, USA). Similar results were obtained in three independent experiments.

cytotoxic to MCF-7 cells. Figure 1 illustrates the effects of 4 nM estradiol, 1 mM DFMO or 1 μM CGP 48664 on DNA synthesis, as determined by the [^{3}H]thymidine incorporation assay on the cells at 16 h after initiation of the cell cycle. This time point in the cell cycle was chosen because flow cytometric measurements showed it to represent transition of G1 cells to the S phase, where critical estrogenic signals interact with mediators of cell cycle progression (Thomas & Thomas 1994, Thomas *et al*. 1997). Estradiol induced a 2-fold increase in DNA synthesis compared with control cells. DFMO and CGP 48664 inhibited [^{3}H]thymidine incorporation in the absence of estradiol. An estradiol-induced increase in [^{3}H]thymidine incorporation was not observed in cells treated with DFMO. In cells treated with CGP 48664, estradiol caused a 2-fold increase in [^{3}H]thymidine incorporation compared with cells treated with CGP 48664 alone; however, this level was only 50% of the level in control cells. Thus polyamine biosynthesis appears to be critical to cell proliferation in both the presence and absence of estradiol, although a muted response to estradiol is produced in cells treated with CGP 48664.

Figure 2 shows the results of our PAGE analysis of ER-associated proteins in cells during the G1/S transition of the cell cycle. In control cells, only two proteins of molecular masses 25 and 45 kDa were associated with GST-ER, whereas ten proteins (estimated molecular masses, 300, 180, 160, 140, 100, 60, 45, 30, 28 and 25 kDa) were associated with GST-ER in cells treated with 4 nM estradiol. The intensity of the 45 kDa protein was 2-fold higher than that of control cells. In the absence of estradiol, DFMO-treated cells showed a low level of ER association with these proteins, but the 45 kDa protein was more intense than others. When cells were treated with DFMO and estradiol, only one major band corresponding to the 25 kDa protein was detected. Interestingly, similar results were obtained in cells treated with CGP 48664. These results suggest a requirement for polyamines and estradiol for optimum association of proteins with ER in the estrogenic pathway of signal transduction.

A set of control experiments was conducted using the GST expression vector without the region encoding ER. Analysis of GST-associated proteins showed the presence of only a 25 kDa protein (results not shown). Thus the 25 kDa protein of Fig. 2 appears to be unrelated to ER function.

Table 1 shows a quantification of total proteins, [^{35}S]methionine-labeled proteins and

TABLE 1. Quantification of total, [^{25}S]methionine-labeled and ER-associated proteins in MCF-7 cells after treatment with DFMO and CGP 48664. Cells were treated with DFMO (1 mM) or CGP 48664 (1 μM) as described in Materials and Methods. Cells were metabolically labeled by [^{25}S]methionine and harvested 16 h after initiation of the cell cycle. Results are the mean and standard deviations from three different experiments

	Total protein (mg)	**Total c.p.m.** ($\times 10^8$)	**GST-ER eluate** (c.p.m. 10^6)
Treatment			
Control	16·2 ± 1·8	3·75 ± 0·23	1·12 ± 0·12
Estradiol	18·0 ± 2·1	5·55 ± 0·42	5·55 ± 0·22
DFMO	20·1 ± 2·3	3·27 ± 0·33	2·99 ± 0·23
DFMO+estradiol	16·3 ± 1·6	2·85 ± 0·24	1·09 ± 0·13
CGP 48664	20·1 ± 1·7	3·30 ± 0·25	3·37 ± 0·25
CGP 48664+estradiol	20·2 ± 2·0	3·45 ± 0·22	1·30 ± 0·10

TABLE 2. Effect of DFMO and CGP 48664 on polyamine levels in MCF-7 cells. Cells were treated with DFMO (1 mM) or CGP 48664 (1 μM) as described under Materials and Methods. Polyamine levels were determined using an HPLC technique from cells harvested at 16 h after initiation of the cell cycle. Restuls are the mean ± S.E. from two triplicate experiments

	Polyamine concentrations (nmol/mg protein)		
	Putrescine	Spermidine	Spermine
Treatment			
Control	0·73 ± 0·03	5·50 ± 0·11	5·20 ± 0·38
Estradiol	0·80 ± 0·09	5·18 ± 0·27	6·26 ± 0·15
DFMO	0·28 ± 0·08[a]	1·20 ± 0·03[a]	4·97 ± 0·29
DFMO+estradiol	0·39 ± 0·06[a]	1·16 ± 0·07[a]	4·80 ± 0·08
CGP 48664	4·45 ± 0·36[a]	2·56 ± 0·14[a]	3·27 ± 0·23[a]
CGP 48664+estradiol	5·28 ± 0·40[a]	2·77 ± 0·06[a]	3·38 ± 0·08[a]

[a]Significantly different from controls ($P<0·05$; Student's *t*-test).

GST-ER-associated proteins. Total protein concentration was determined by the Bradford protein assay (Bradford 1976). Radioactivity of the cellular lysate as well as that of the eluate from GST-ER–Sepharose beads was used to quantify newly synthesized and ER-associated proteins respectively. Our results showed that the total protein level in different treatment groups was not significantly different. [^{35}S]methionine-labeled proteins in estradiol-treated cells were higher than those in all other groups, as expected from the overall mobilization of DNA replication and protein synthesis machinery by estradiol. Although the amount of total [^{35}S]methionine-labeled proteins was only 2-fold higher in estradiol-treated cells than in controls, a 5-fold increase in the radioactivity of ER-associated proteins was found. There was no significant difference in [^{35}S]methionine-labeled proteins between the DFMO treatment group and the DFMO+estradiol treatment group. However, there was a 3-fold decrease in the level of ER-associated proteins in the presence of DFMO+estradiol compared with that of the DFMO treatment group. Results on CGP 48664 treatment were similar to that of DFMO. These results suggest that differences observed between treatment groups are mainly due to changes in the ability of the proteins to associate with ER, rather than changes in protein synthesis.

Table 2 shows the effect of DFMO and CGP 48664 on intracellular polyamine levels at 16 h after initiation of the cell cycle. Estradiol did not significantly increase the level of polyamines compared with the controls at this time point after initiation of the cell cycle. Therefore, the estradiol-induced increase in the level of ER association of proteins is due to ligand-dependent changes in ER. DFMO treatment caused a decrease in the level of putrescine and spermidine, without significant changes in spermine concentration. Treatment of cells with CGP 48664 reduced spermidine and spermine levels; however, it increased putrescine concentrations. Compensatory increases in putrescine after treatment with CGP 48664 have been previously reported (Regenass *et al*. 1994, Thomas *et al*. 1996). However, this compensatory increase is

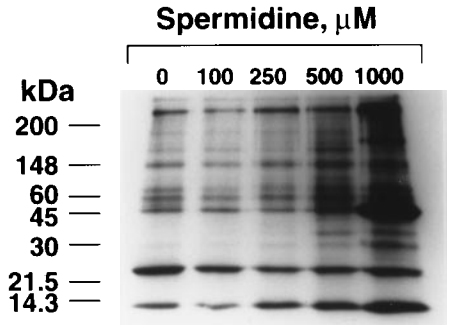

FIGURE 3. Effect of spermidine on ER association of proteins. Cellular lysate samples (100 μl) from estradiol+DFMO treatment group were incubated with 0, 100, 250, 500 and 1000 μM spermidine at 4 °C for 90 min. The samples were then treated with GST-ER-loaded glutathione–Sepharose for 16 h and eluted with 20 mM glutathione. Samples were then analyzed by SDS PAGE and subjected to autoradiography.

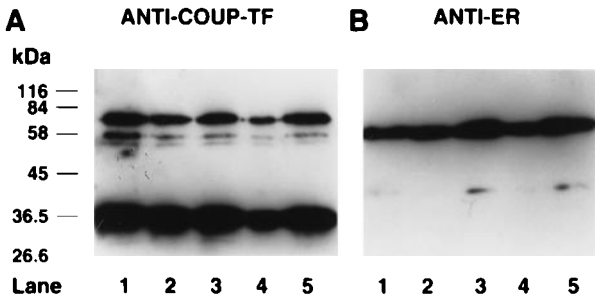

FIGURE 4. Western blot analysis of the sample set shown in Fig. 3 using anti-COUP-TF and anti-ER antibodies. Lanes 1–5 represent 0, 100, 250, 500 and 1000 μM spermidine. The blot was first probed with anti-COUP-TF antibody, then stripped and reprobed with anti-ER antibody.

insufficient to fully support DNA synthesis (Fig. 1). Similar changes in ER association of proteins were induced by both DFMO and CGP 48664 (Fig. 2). Since reduction of spermidine was the only common feature in the action of these two inhibitors, spermidine appears to be a critical factor in the modulation of ER binding to other proteins.

In order to examine whether the effect of DFMO on the level of ER-associated proteins was due to changes in the pattern of expression of proteins or due to changes in protein–protein association due to polyamine depletion, we performed the following experiment. Aliquots of cell lysate from the estradiol+DFMO treatment group were incubated with 100, 250, 500 and 1000 μM concentrations of spermidine and then analyzed by the GST-ER pull down assay. Figure 3 shows our results. Incubation with 100–1000 μM spermidine caused a dramatic increase in the association of several proteins (estimated molecular masses, 300, 180, 160, 140, 100, 80, 60, 45, 35, 30 and 25 kDa). The specific protein retained with the highest intensity was a 45 kDa protein. Interestingly, two proteins of estimated molecular masses 100 and 160 kDa increased in intensity at 500 μM spermidine, but decreased at 1000 μM spermidine. In contrast, the 25 kDa protein that appeared to bind to the GST part of the fusion protein showed no significant change with spermidine concentration and hence it would serve as an internal control for loading in each lane. These results show that the reduction of ER association of proteins with DFMO is mostly reversible by the addition of spermidine. Secondly, in the presence of an equal amount of [^{35}S]methionine-labeled proteins, reaction mixtures containing different concentrations of spermidine yielded different levels of ER-associated proteins, demonstrating the importance of spermidine in these protein–protein interactions.

In an attempt to identify ER-associated proteins observed in Figs 2 and 3, we next examined the presence of COUP-TF, a transcription factor known to modulate ER function (Klinge *et al*. 1997) as well as the endogenous ER by Western blot analysis. Figure 4 shows the results of our Western blot analysis of a sample set identical to that used in Fig. 3. Anti-COUP-TF and anti-ER antibodies were used as probes in the Western blot. A high-intensity protein, immunoreactive to COUP-TF (36 kDa), was identified in the Western blot (Fig. 4A). Additional bands showing low immunoreactivity (78, 58 and 55 kDa) were also present. Whether these represent nonspecific binding or a reactivity of other proteins homologous to COUP-TF is not known at this time. Interestingly, the level of COUP-TF binding in the presence and absence of exogenous spermidine was similar. This would suggest that COUP-TF binding to ER is not regulated by spermidine, although it is pulled down by GST-ER. The high level of immunoreactive protein in the absence of corresponding [^{35}S]methionine-labeled 36 kDa protein suggests that COUP-TF is constitutively present in the G1/S phase cells and that little new synthesis of this protein is occurring during the 4 h time period of exposure of these cells to [^{35}S]methionine.

Western blot analysis using anti-ER antibody showed only one band of approximately 60 kDa as expected for full-length ER protein. The level of endogenous ER associated with the GST-ER increased by 2-fold at 250 and 1000 μM spermidine, but the control level was found at 500 μM

spermidine. (This decrease appears to be due to low level of protein in lane 4.) Thus, GST-ER association with endogenous ER is sensitive to the level of polyamines in the reaction mixture. This would also suggest that the 60 kDa protein in Figs 2 and 3 is endogenous ER.

DISCUSSION

Our studies show the association of nine cellular proteins with ER in estradiol-treated MCF-7 cells. In contrast, only one 45 kDa protein was specifically associated with ER in control cells. In cells treated with DFMO in the absence of estradiol, multiple proteins were associated with ER at a low level, but the 45 kDa protein was prominent. In the presence of DFMO and estradiol, ER association of proteins decreased, although the nonspecific association of the 25 kDa protein was unchanged. The remarkable up-regulation of ER association of proteins in the presence of estradiol, the lack of these associations in the presence of estradiol and DFMO, and the reversibility of most of the DFMO effects by exogenous spermidine indicate a critical role for this polyamine in ER–protein associations and estrogen-induced signal transduction pathways in breast cancer cells. Although COUP-TF and endogenous ER were identified as ER-associated proteins, an uncharacterized 45 kDa protein appears to be a major regulator of polyamine action in ER function. Ligand-induced conformational changes in ER may allow its association with specific proteins either as a multi-protein complex or in a sequential manner. The involvement of polyamine biosynthesis in this process indicates a fine tuning of protein–protein interactions as a requirement for estrogenic regulation of gene expression and cell growth. There was an increase in ER association of proteins in the presence of DFMO alone compared with controls without estradiol treatment, although the level of binding is much lower than that observed in the presence of estradiol. This is in contrast to a decrease in binding observed in the estradiol+DFMO group. A plausible explanation for the association of multiple proteins in the DFMO group is that polyamines might inhibit the interaction of unliganded ER with these proteins, and DFMO increases these interactions by depleting polyamines. However, in the presence of estradiol, multiple proteins require polyamines for optimal association with liganded ER. Thus a possible mechanism might involve the inhibition of low-affinity protein–protein interactions by polyamines, while facilitating high-affinity interactions.

Previous studies have shown that the maximal increase in polyamine levels after estradiol treatment occurred at 8 h after initiation of the cell cycle from G1 phase (Thomas & Thomas 1993). The peak levels of polyamines decreased during the 12–24 h time period. In the absence of estradiol, a moderate increase in polyamines was observed by 12 h after initiation of the cell cycle (Thomas & Thomas 1993). Therefore, little difference in polyamines was observed between control and estradiol-treated samples at the 16 h time point. Thus the current results are consistent with our previous observations.

Several recent reports demonstrate the importance of protein–protein interactions in ER-mediated signal transduction pathways. Halachmi *et al*. (1994) first provided evidence for the association of ER with a novel protein of 160 kDa, using a GST-ER pull down assay. Later studies by this group (Hanstein *et al*. 1996) identified a number of proteins from 30 to 300 kDa which associated with ER. Among these proteins, p300 was characterized as histone acetylase and an adaptor for several nuclear receptors and cAMP response factor, thus serving as an integrator of multiple signal transduction pathways. We found the presence of a 300 kDa band (Fig. 3) in our GST-ER pull down assay and its level was significantly increased by exogenous spermidine.

Landel *et al*. (1994) reported the presence of ER-associated proteins in ER purified through immuno-, steroid- and site-specific DNA-affinity chromatography. Proteins of 70, 55, 48 and 45 kDa were detected in SDS gels by silver staining. The 70 kDa protein was subsequently identified as a heat shock protein (hsp70). N-terminal microsequencing indicated that the 55 kDa protein was a member of the protein disulfide isomerase family. The 48 and 45 kDa proteins were not identified. A report by Cavailles *et al*. (1994) using a GST-ER pull down assay identified 160, 140 and 80 kDa proteins. Zeiner & Gehring (1995) cloned the cDNA for ER-associated 45 kDa protein. Other candidates for the 45 kDa protein include casein kinase II and cyclin D1, as these proteins have also been reported to associate with ER.

It is now recognized that the mechanism of action of nuclear receptors is interlinked by the presence and interactions of co-activators and/or co-repressors. For example, the 125 kDa steroid receptor co-activator-1 protein interacts with estrogen and progesterone receptors and stimulates the transcriptional activity of these and other steroid hormone receptors (Onate *et al*. 1995). Several families of co-activators that associate with steroid receptors and their encoding genes have been

isolated from yeast, mouse and human cDNA libraries (Horwitz *et al*. 1996). It is not as yet clear how these protein–protein interactions are integrated by the cell to result in a measured response in terms of the transcriptional activation. The ability of a histone acetyltransferase or p300 to associate with ER and many other sequence-specific transcriptional activators appears to serve as a common integration point (Hanstein *et al*. 1996, Ogryzko *et al*. 1996). Our results suggest that intracellular polyamine levels might be another important factor in determining the extent of protein–protein interactions and transcriptional responses. Our studies on the interaction of polyamines with ER by physical chemical methods indicate that polyamines are able to provoke structural/conformational changes in ER and alter its ligand dissociation kinetics (Thomas & Kiang 1987*b*). More recently, analysis of polyamine-induced conformational changes in peptides using circular dichroism spectroscopy indicates that site-specific polyamine binding leads to changes in the α-helical content of the peptide (Tabet *et al*. 1993). Thus changes in the conformation of ER or that of other proteins might be important in the association of proteins with ER and downstream events regulated by ER.

The mechanism of action of steroid receptors includes the involvement of protein kinases in transcriptional activation. Activation of ER through mitogen-activated protein kinase (Kato *et al*. 1995) and the identification of ER phosphorylation at tyrosine 537 and serine 167 (Arnold *et al*. 1994, 1995) are pertinent. Purified ER has also been found to be associated with casein kinase II (Arnold *et al*. 1994). Recently, cyclin D1 was found to be associated with ER (Zwijsen *et al*. 1997). Thus estrogenic regulation of gene expression and cell growth appears to involve the interaction of ER with several kinases, transcriptional co-activators and repressors, as well as cell cycle regulatory proteins. GST-ER pull down assays detect possible interactions between ER and cellular proteins. Reduction of polyamine levels alters the association of ER with cellular proteins such that estradiol appears to be unable to activate gene expression and facilitate cell growth. Changes in the phosphorylation status of ER or other proteins may also contribute to the decrease in protein–protein interactions, since polyamines are known to increase the activity of casein kinase II (Cochet & Chambez 1983, Leroy *et al*. 1997). However, exogenous addition of spermidine, prior to GST pull down assays, was able to restore ER association of proteins, suggesting that the differences in ER-associated proteins observed in our studies are mostly due to changes in the level of spermidine.

In summary, our studies indicate that polyamines are critical to the regulation of ER association of other proteins. A decrease in cellular polyamine levels suppressed estradiol-induced increases in the association of ER with cellular proteins. Addition of exogenous polyamines increased protein–protein interactions involving ER. These results support a functional link between polyamines and estrogenic function in gene expression and cell proliferation.

ACKNOWLEDGEMENTS

This work was supported by NIH Grants, CA 42439 (T T), CA 73058 (T J T) and DK 53220 (C M K) and the NIEHS Center of Excellence (ES 05022, M A G).

REFERENCES

Arnold SF, Obourn JD, Jaffe H & Notides AC 1994 Serine 167 is the major estradiol-induced phosphorylation site on the hER. *Molecular Endocrinology* **8** 1208–1214.

Arnold SF, Obour JD, Jaffe H & Notides AC 1995 Phosphorylation of the human estrogen receptor on tyrosine 537 *in vivo* and by *Src* family tyrosine kinases *in vitro*. *Molecular Endocrinology* **9** 24–33.

Beato M, Herrlich P & Schütz G 1995 Steroid hormone receptors – many actors in search of a plot. *Cell* **83** 851–857.

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.

Cavailles V, Dauvois S, Danielian PS & Parker MG 1994 Interaction of proteins with transcriptionally active estrogen receptors. *Proceedings of the National Academy of Sciences of the USA* **91** 10009–10013.

Cochet C & Chambez EM 1983 Polyamine-mediated protein phosphorylations: A possible target for intracellular polyamine action. *Molecular and Cellular Endocrinology* **30** 247–266.

Faaland CA, Laskin JD & Thomas TJ 1995 Inhibition of epidermal growth factor-stimulated EGF receptor tyrosine kinase activity in A431 human epidermoid carcinoma cells by polyamines. *Cell Growth and Differentiation* **6** 115–121.

Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ & West BL 1998 Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* **280** 1747–1749.

Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C & Brown M 1994 Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* **264** 1455–1458.

Hanstein B, Eckner R, DiRenzo J, Halachmi S, Liu H, Searcy B, Kurokawa R & Brown M 1996 p300 is a component of an estrogen receptor coactivator complex. *Proceedings of the National Academy of Sciences of the USA* **93** 11540–11545.

Heby O & Persson L 1990 Molecular genetics of polyamine synthesis in eukaryotic cells. *Trends in Biochemical Sciences* **15** 153–158.

Horwitz KB, Jackson DL, Richer GS & Tung L 1996 Nuclear receptor coactivators and corepressors. *Molecular Endocrinology* **10** 1167–1177.

Hu RH & Pegg AE 1997 Rapid induction of apoptosis by deregulated uptake of polyamine analogues. *Biochemical Journal* **328** 307–316.

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D & Chambon P 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270** 1491–1494.

Katzenellenbogen JA, O'Malley BW & Katzenellenbogen BS 1996 Tripartite steroid hormone receptor pharmacology: interaction with multiple effector sites as a basis for the cell and promoter-specific action of these hormones. *Molecular Endocrinology* **10** 119–131.

Klinge CM, Silver BF, Driscoll MD, Sathya G, Bambara RA & Hilf R 1997 Chicken ovalbumin upstream promoter-transcription factor interacts with estrogen receptor, binds to estrogen response elements and half-sites, and inhibits estrogen-induced gene expression. *Journal of Biological Chemistry* **272** 31465–31474.

Landel CC, Kushner PJ & Greene GL 1994 The interaction of human estrogen receptor with DNA is modulated by receptor-associated proteins. *Molecular Endocrinology* **8** 1407–1419.

Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J & Hilsenbeck SG 1998 Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proceedings of the National Academy of Sciences of the USA* **95** 2920–2925.

Leroy D, Filhol O, Delcros JG, Pares S, Chambaz EM & Cochet C 1997 Chemical features of the protein kinase CK2 polyamine binding site. *Biochemistry* **36** 1242–1250.

Manni A 1989 Polyamines and hormonal control of breast cancer cell growth. *CRC Critical Reviews in Oncogenesis* **1** 163–174.

Manni A & Wright C 1984 Polyamines as mediators of estrogen action on the growth of experimental breast cancer in rats. *Journal of the National Cancer Institute* **73** 511–514.

McCloskey DE, Yang J, Woster NE, Davidson NE & Casero RA Jr 1996 Polyamine analog induction of programmed cell death in human lung tumor cells. *Clinical Cancer Research* **2** 441–446.

Metcalf BW, Bey P, Danzin C, Jung MJ, Casara P & Velvet JP 1978 Catalytic irreversible inhibition of mammalian ornithine decarboxylase. *Journal of the American Chemical Society* **100** 2551–2553.

Moshier J, Dosescu J, Skunca M & Luk GD 1993 Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression. *Cancer Research* **53** 2618–2622.

Ogryzko VV, Schiltz RL, Russanova V, Howard BH & Nakatani Y 1996 The transcriptional co-activators p300 and CBP are histone acetyltransferases. *Cell* **87** 953–959.

Onate SA, Tsai SY, Tsai MJ & O'Malley BW 1995 Sequence and characterization of a co-activator for the steroid hormone receptor superfamily. *Science* **270** 1354–1357.

Poulin R, Coward JK, Lakanen JR & Pegg AE 1993 Enhancement of the spermidine uptake system and lethal effects of spermidine overaccumulation in ornithine decarboxylase-overproducing L1210 cells under hypoosmotic stress. *Journal Biological Chemistry* **268** 4690–4698.

Regenass U, Mett H, Stanek J, Müeller M, Kramer D & Porter CW 1994 CGP 48664, a new *S*-adenosylmethionine decarboxylase inhibitor with broad spectrum antiproliferative and antitumor activity. *Cancer Research* **54** 3210–3217.

Smith MK, Trempus CS & Gilmour SK 1998 Co-operation between follicular ornithine decarboxylase and v-Ha-ras induces spontaneous papillomas and malignant conversion in transgenic skin. *Carcinogenesis* **19** 1409–1415.

Tabet M, Labroo V, Sheppard P & Sasaki T 1993 Spermine induced conformational changes of a synthetic peptide. *Journal of the American Chemical Society* **115** 3866–3869.

Tabib A & Bachrach U 1994 Activation of the proto-oncogene c-*myc* and c-*fos* by c-*ras*: involvement of polyamines. *Biochemical and Biophysical Research Communications* **202** 720–727.

Tabor CW & Tabor H 1984 Polyamines. *Annual Reviews in Biochemistry* **53** 749–790.

Thomas T & Kiang DT 1987*a* Additive growth-inhibitory effects of difluoromethylornithine and antiestrogens on MCF-7 cell line. *Biochemical and Biophysical Research Communications* **148** 1338–1345.

Thomas T & Kiang DT 1987*b* Structural alterations and stabilization of rabbit uterine estrogen receptors by natural polyamines. *Cancer Research* **47** 1799–1804.

Thomas T & Thomas TJ 1993 Estradiol control of ornithine decarboxylase mRNA, enzyme activity, and polyamine levels in MCF-7 breast cancer cells: therapeutic implications. *Breast Cancer Research and Treatment* **29** 189–201.

Thomas T & Thomas TJ 1994 Regulation of cyclin B1 by estradiol and polyamines in MCF-7 breast cancer cells. *Cancer Research* **54** 1077–1084.

Thomas T, Gallo MA, Klinge CM & Thomas TJ 1995 Polyamine-induced conformational perturbations in DNA alter the binding of estrogen receptor to poly(dG-m^{5}dC). poly(dG-m^{5}dC) and a plasmid containing estrogen response element. *Journal of Steroid Biochemistry and Molecular Biology* **54** 89–99.

Thomas T, Faaland CA, Adhikarakunnathu S & Thomas TJ 1996 Structure–activity relations of S-adenosylmethionine decarboxylase inhibitors on the growth of MCF-7 breast cancer cells. *Breast Cancer Research and Treatment* **39** 293–306.

Thomas TJ, Adhikarakunnathu S, Faaland CA & Thomas T 1997 Estrogenic induction of a cyclin B1 associated protein at the G1 to S phase transition. *Oncology Reports* **4** 15–21.

Zeiner M & Gehring U 1995 A protein that interacts with members of the nuclear hormone receptor family: identification and cDNA cloning. *Proceedings of the National Academy of Sciences of the USA* **92** 11465–11469.

Zwijsen RML, Wientjens E, Klompmaker R, van der Sman J, Bernards R & Michailides JAM 1997 CDK independent activation of estrogen receptor by cyclin D1. *Cell* **88** 405–415.

REVISED MANUSCRIPT RECEIVED 27 August 1998