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Early estrogen-induced metabolic changes and their inhibition by actinomycin D and cycloheximide in human breast cancer cells: ^{31}P and ^{13}C NMR studies

(tamoxifen/glucose metabolism)

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ABSTRACT **Metabolic changes following estrogen stimulation and the inhibition of these changes in the presence of actinomycin D and cycloheximide were monitored continuously in perfused human breast cancer T47D clone 11 cells with ^{31}P and ^{13}C NMR techniques. The experiments were performed by estrogen rescue of tamoxifen-treated cells. Immediately after perfusion with estrogen-containing medium, a continuous enhancement in the rates of glucose consumption, lactate production by glycolysis, and glutamate synthesis by the Krebs cycle occurred with a persistent 2-fold increase at 4 hr. The content of phosphocholine had increased by 10% to 30% within the first hour of estrogen stimulation, but the content of the other observed phosphate metabolites as well as the pH remained unchanged. Pretreatment with either actinomycin D or cycloheximide, at concentrations known to inhibit mRNA and protein synthesis, respectively, and simultaneous treatment with estrogen and each inhibitor prevented the estrogen-induced changes in glucose metabolism. This suggested that the observed estrogen stimulation required synthesis of mRNA and protein. These inhibitors also modulated several metabolic activities that were not related to estrogen stimulation. The observed changes in the *in vivo* kinetics of glucose metabolism may provide a means for the early detection of the response of human breast cancer cells to estrogen versus tamoxifen treatment.**

Estrogens play a major role in the pathogenesis of breast cancer. Human breast cancer cell lines that retain receptors for estrogen have provided a useful model system for the study of hormonal responses (1). Treatment of these cells with estrogen enhanced cell proliferation, whereas treatment with antiestrogens resulted in an arrest of cell division (2). Estrogen stimulation in breast cancer cells was also expressed in enhanced synthesis of proteins and increased enzymatic activities (refs. 1 and 3 and references therein). Most of these changes were measured after several days of estrogen treatment, and only few of the metabolic activities enhanced within the first 24 hr after estrogen administration were characterized (1, 4, 5).

The recently developed methods for monitoring the metabolism of actively growing cancer cells in culture (6–8) with NMR techniques have provided an additional tool for noninvasively characterizing estrogen-induced metabolic changes. Variations in the content of the phosphate metabolites and in glucose metabolism were found between T47D human breast cancer cells cultured for 5 days with estrogen versus those cultured for 5 days with the antiestrogen drug tamoxifen (3). Here we present ^{31}P and ^{13}C NMR studies of the early estrogen-induced metabolic changes in perfused T47D clone 11 human breast cancer cells. The changes with time in the content of the phosphate metabolites, in the rate of glucose utilization, and in the rates of lactate and glutamate synthesis were monitored continuously during the first 24 hr after estrogen rescue of tamoxifen-treated cells. A similar experimental scheme of antiestrogen rescue was previously designed to emphasize the effects of estrogen stimulation (9). In this scheme, cells were pretreated for a few days with antiestrogen agents such as tamoxifen to inhibit the effect of residual estrogens present in charcoal-treated serum (10) and of compounds with estrogenic activity present in the medium (11). It has been shown that during long-term antiestrogen treatment estrogen-responsive human breast cancer cells are arrested in the G_1 phase of the cell cycle and, after estrogen administration, a partial synchronization of the first cell division is obtained (12).

The mechanism of estrogen induction can be investigated by employing inhibitors such as actinomycin D and cycloheximide. Actinomycin D has complex functions, but it can be added at a dose that is known to inhibit mRNA synthesis (13). Cycloheximide inhibits ribosome-dependent protein synthesis (14). The effects of these inhibitors on estrogen binding and on several estrogen-induced processes were previously studied in human breat cancer cell lines by using biochemical methods (15–17). We present here results of the use of these inhibitors for verifying the mechanism of stimulation of glucose metabolism by estrogen in T47D clone 11 cells. In addition, information was obtained regarding the effects of the inhibitors themselves on the energetics and on glucose metabolism in these cells.

MATERIALS AND METHODS

Cell Culture. T47D clone 11 cells were cultured on agarose-polyacrolein microcarrier beads (Galisar, Ramat Gan, Israel) coated with polylysine as described (6, 7). Two days after seeding, the beads with the cells were washed in phenol red-free Dulbecco's modified Eagle's medium (DMEM). Subsequently culturing was continued with phenol red-free DMEM supplemented with 10% fetal calf serum (treated with dextran-coated charcoal) and 2×10^{-6} M tamoxifen citrate (Sigma) (3). After 5 days of culture in the presence of tamoxifen, 2.5 ml of beads with cells were placed in a 10-mm NMR tube and perfused under sterile conditions with phenol red-free DMEM plus 10% charcoal-treated fetal calf serum oxygenated with 95% O_2/5% CO_2 by using a system described in detail elsewhere (3). The number of cells per volume of beads in each experiment was determined by counting duplicate samples after harvesting the cells from the beads by trypsinization.

Estrogen (17β-estradiol) was dissolved in ethanol (1 mg/ml) and diluted in the perfusion medium to a final concentration of 3×10^{-8} M. Tamoxifen was added to the perfusion medium at a concentration of 2×10^{-6} M. Cycloheximide (1 mg/ml) was dissolved in phosphate-buffered saline and di-

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luted in the perfusion medium to a final concentration of 10 μg/ml. Actinomycin D was dissolved in ethanol (2.5 mg/ml) and diluted to a final concentration of 2.5 μg/ml. The cytotoxic effect of cycloheximide and actinomycin D on T47D clone 11 cells was studied in monolayer culture by trypan blue staining. No enhanced cell death was observed during 24 hr of incubation with medium containing either cycloheximide at 10 μg/ml or actinomycin D at 2.5 μg/ml.

NMR Measurements. NMR measurements were performed on a Bruker AM-500 spectrometer by using a multinuclear probe for which detection of four nuclei (^{31}P, ^{13}C, ^{15}N, and ^{1}H) can be selected by a software command. Proton-decoupled, fully relaxed ^{31}P NMR spectra were recorded at 202.5 MHz by applying 90° pulses, 10-sec repetition time, and continuous composite pulse proton decoupling (1 W). The ^{31}P signals were previously assigned (18). The content of phosphate metabolites was determined in reference to the signal area of the medium P_i (125 mg/liter) as described (3).

^{13}C NMR spectra were recorded at 125.7 MHz by applying 60° pulses, 6-sec repetition time, and composite pulse proton decoupling (1 W) (3). The ^{13}C signals were assigned previously (3). Glucose labeled at position 1 (99% ^{13}C; MSD Isotopes) was added at 1 mg/ml to the perfusate (glucose-free, phenol red-free DMEM plus 10% charcoal-treated fetal calf serum and hormones as indicated) at time 0, and 15-min spectra (150 scans) were accumulated sequentially for 3–4 hr. Before initiating the next ^{13}C time course experiment on the same sample, the ^{13}C label was washed away by perfusing the cells for the same period (3–4 hr) with medium containing unlabeled glucose (1 mg/ml). At the end of this period, a ^{13}C spectrum was taken to monitor the extent of label disappearance. Areas of the signals were scaled in reference to the medium glucose at time 0 (5.6 mM) (3). Further details regarding analysis of the ^{13}C data are described in ref. 3.

RESULTS

The experiments were designed to characterize and monitor the early estrogen-induced metabolic changes in perfused T47D clone 11 human breast cancer cells cultured on microcarrier beads. The cells, treated in the incubator for 5 days with tamoxifen, were perfused in the NMR spectrometer tube with the same tamoxifen-containing medium, and then estrogen rescue was initiated by perfusing the cells with estrogen-containing medium. The experimental protocols included alternate ^{31}P and ^{13}C measurements in the presence of tamoxifen or estrogen and glucose with and without ^{13}C enrichment (Fig. 1A). The composition and content of the phosphate metabolites were determined from ^{31}P NMR spectra. Within the first 2 hr of perfusion with estrogen, the content of phosphocholine increased by about 18% (ranging between 10% and 33% in five experiments) and remained constant for at least 20 hr. The content per cell of the other phosphates—nucleoside triphosphates, uridine diphosphate sugar, phosphocreatine, glycerolphosphocholine, and glycerolphosphoethanolamine—did not change (within experimental error) following estrogen administration.

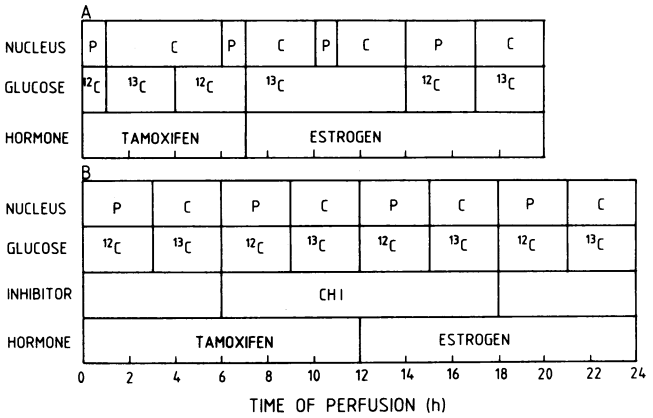

FIG. 1. Experimental protocols designed for studying the early estrogen effects on phosphate and glucose metabolism. T47D clone 11 cells were cultured on beads for 5 days in the presence of tamoxifen and prepared for NMR experiments. (*A*) A representative protocol designed to study the transition from tamoxifen to estrogen. (*B*) A representative protocol designed to study estrogen effects in the presence of inhibitors, such as cycloheximide (CHI). The time intervals varied between experiments.

The rates of glucose consumption, lactate production, and glutamate synthesis were determined by ^{13}C NMR measurements of cells perfused with medium containing [1-^{13}C]glucose. Estrogen administration induced a highly significant increase (*t* test, $P < 0.005$; Table 1, experiment A) in the rate

Table 1. Effects of estrogen and inhibitors on lactate production, glucose consumption, and glutamate synthesis

				^{13}C flux, fmol per cell per hr					
				Lactate		Glutamate		Glucose	
Exp.	Treatment	Measurement interval, hr	*n*	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
A	Tamoxifen	0–4*	7	250 ± 30	210–350	2.3 ± 0.2	1.9–3.0	260 ± 40	210–280
	Estrogen	0–5†	4	480 ± 40	420–530	4.1 ± 0.2	4.0–4.2	530 ± 50	460–540
	Estrogen	8–17†	4	560 ± 20	520–580	5.3‡	5.0–5.5	570 ± 30	520–600
B	Tamoxifen + Act D	4–8§	2	330	300–350	1.5	1.4–1.5	330	300–360
	Estrogen + Act D	4–8¶	2	330	310–350	0.8	0.7–0.8	340	320–360
	Estrogen − Act D	4–8‖	2	310	300–320	0.4	0.2–0.5	320	300–340
C	Tamoxifen + CHI	3–6§	2	160	120–200	1.1	1.0–1.2	240	230–250
	Estrogen + CHI	3–6¶	2	160	130–190	0.9	0.6–1.2	240	230–240
	Estrogen − CHI	3–6‖	2	240	220–260	1.9	1.8–2.0	250	220–270

Act D, actinomycin D; CHI, cycloheximide.

*Obtained during the first 4 hr after initiation of measurements as shown in Fig. 1A. The cells were treated with tamoxifen for 5 days prior to the measurments.

†Obtained during the indicated time interval after changing from medium with tamoxifen to medium with estrogen as shown in Fig. 1A.

‡Results of two experiments. In the other two experiments the medium contained ^{13}C-labeled glucose throughout the time indicated, and the glutamate pool was saturated and did not change.

§Obtained during the indicated time interval after changing from medium with tamoxifen to medium with tamoxifen + inhibitor as shown in Fig. 1B.

¶Obtained during the indicated time interval after changing from medium with tamoxifen + inhibitor to medium with estrogen + inhibitor as shown in Fig. 1B.

‖Obtained during the indicated time interval after changing from medium with estrogen + inhibitor to medium with estrogen as shown in Fig. 1B.

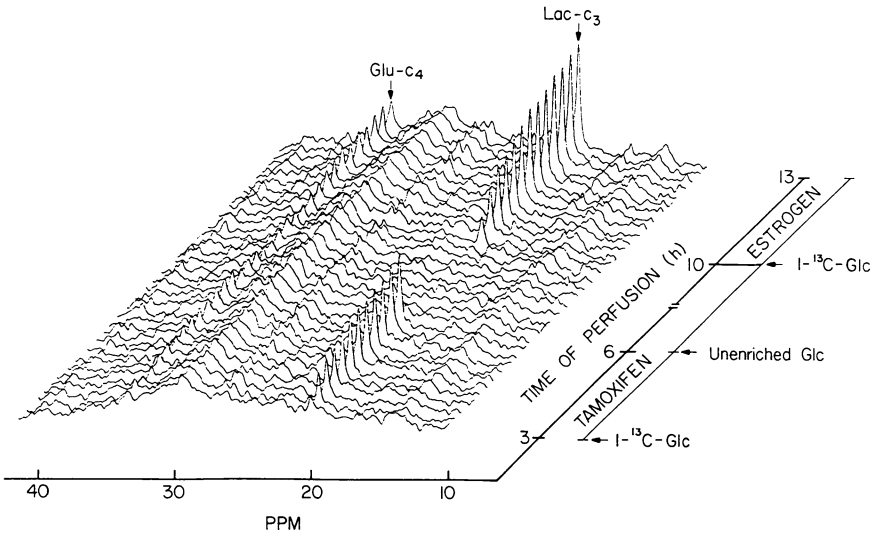

FIG. 2. ^{13}C NMR spectra of T47D clone 11 cells before and after estrogen rescue of tamoxifen-treated cells. Time course of lactate (Lac) and glutamate (Glu) labeling following the addition of [1-^{13}C]glucose (Glc) is shown. Each trace consists of 150 transients.

of glucose consumption, lactate synthesis, and glutamate labeling (Figs. 2 and 3). These changes in the rates were already detected after the first hour of perfusion with estrogen (Fig. 4) and reached an approximately 2-fold maximum constant enhancement 4 hr later.

The mechanism of estrogen induction of glucose metabolism was investigated with the aid of actinomycin D and cycloheximide, at doses known to act as specific inhibitors of mRNA and protein synthesis, respectively. These experiments were performed according to the following two protocols: The first protocol included pretreatment with the inhibitor for 6 hr prior to estrogen addition as shown in Fig. 1*B*. The second protocol was designed to obtain the immediate effects following simultaneous addition of the inhibitor and estrogen. This protocol included duplicate cell samples for each experiment. Both samples were initially perfused for 1 hr with tamoxifen, and then a ^{13}C time course was initiated while adding to one sample tamoxifen plus inhibitor and to a second sample estrogen plus inhibitor. The rates of the two time courses were then compared. Pretreatment with actinomycin D and cycloheximide completely inhibited the estrogen stimulation of glucose metabolism to lactate and glutamate (Table 1). A similar inhibition of the enhancement in glucose metabolism was obtained when estrogen and the inhibitor were added simultaneously. In these experiments the rate of glucose utilization (310 ± 50 fmol per cell per hr), lactate production (300 ± 50 fmol per cell per hr), and glutamate synthesis (2.5 ± 0.5 fmol per cell per hr) remained the same (within experimental error) for cells treated with tamoxifen plus actinomycin D, estrogen plus actinomycin D, tamoxifen plus cycloheximide, and estrogen plus cycloheximide.

These inhibitors also affected the metabolism of these cells irrespective of the presence of tamoxifen or estrogen in the medium: actinomycin-D induced, immediately after its addi-

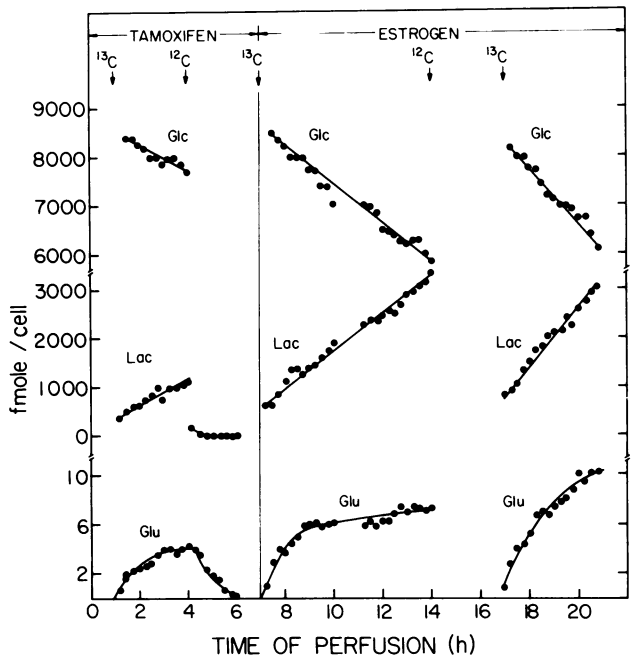

FIG. 3. Time course of glucose (Glc) consumption, lactate (Lac) synthesis, and glutamate labeling (Glu). Data were obtained from sequential 15-min ^{13}C spectra. Glucose concentration was determined from the area of the 1-^{13}C glucose ($\alpha + \beta$) signals. Lactate and glutamate concentration were determined from the area of the $^{13}C_3$ of lactate and $^{13}C_4$ of glutamate.

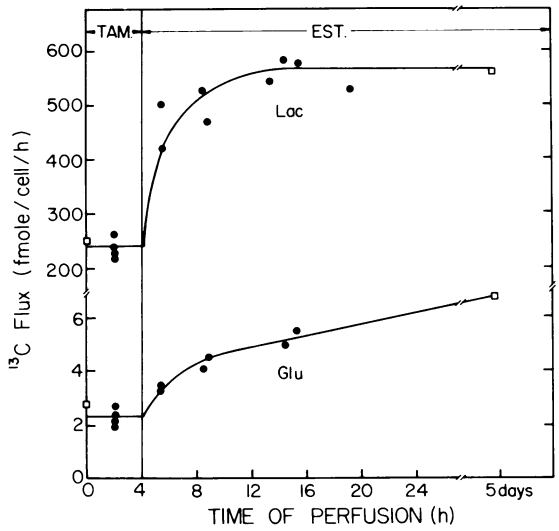

FIG. 4. Time course of estrogen induction of the rates of lactate and glutamate labeling. The rates were calculated from ^{13}C measurements. Cells were treated for 5 days with tamoxifen and induced by estrogen during the NMR experiment (●). Cells were treated for 5 days with either estrogen or tamoxifen (□) (results taken from ref. 3).

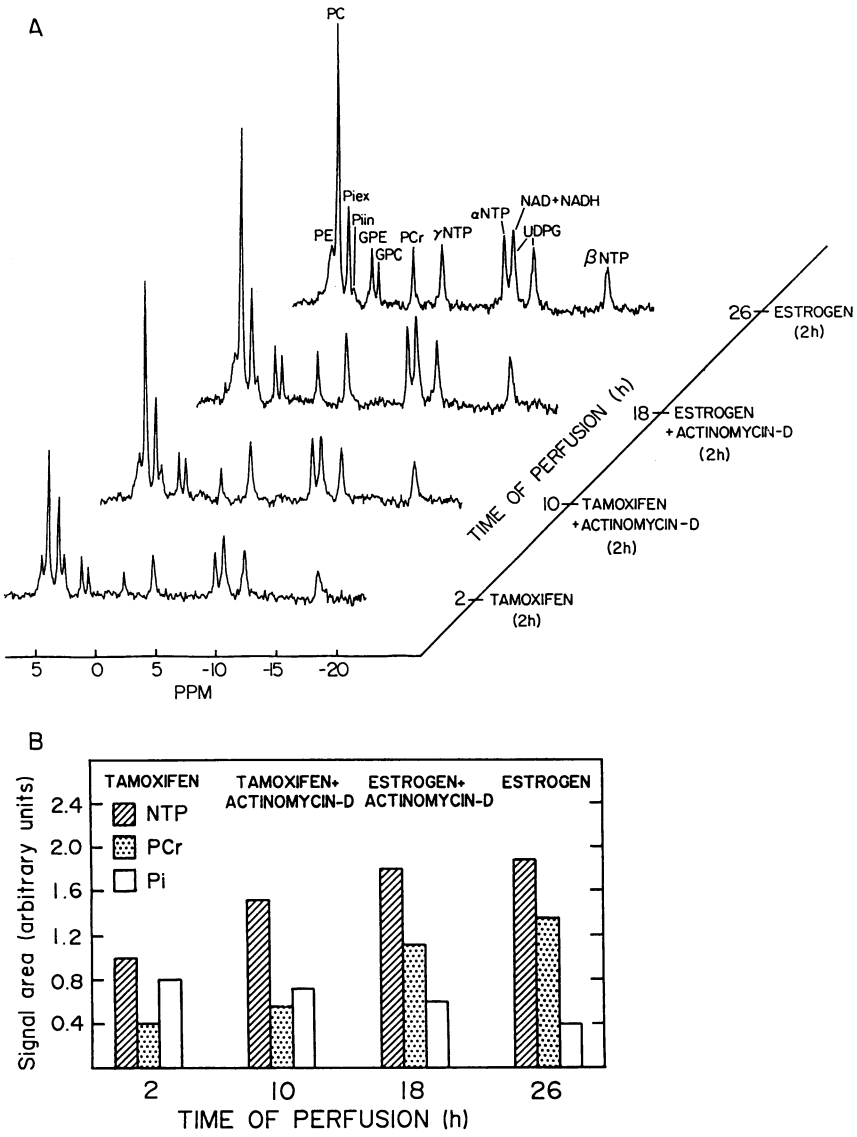

FIG. 5. T47D clone 11 cells perfused with actinomycin D in the presence of either tamoxifen or estrogen. (*A*) ^{31}P spectra obtained from cells cultured for 5 days in the presence of tamoxifen using the protocol given in Fig. 1*B*. (*B*) The increase in nucleoside triphosphates (NTP) and phosphocreatine (PCr) and the decrease in intracellular P_i obtained by analyzing the above spectra. PE, phosphoethanolamine; PC, phosphocholine; GPE, glycerolphosphoethanolamine; GPC, glycerol phosphocholine; UDPG, uridine diphosphate sugar; Piex, external P_i; Piin, internal P_i.

tion, a substantial continuous increase in the content of nucleoside triphosphates and phosphocreatine, with a concomitant decrease in the intracellular content of inorganic phosphate (Fig. 5). Pretreatment with actinomycin D also caused a slight reversible increase (≈20%) in the rate of glucose consumption and lactate production but gradually inhibited the rate of glutamate labeling (Table 1). Experiments with cells, cultured and perfused with DMEM plus 10% fetal calf serum with no specific treatment with either estrogen or tamoxifen, showed a similar response to actinomycin D: a transient accumulation of nucleoside triphosphates for ≈10 hr and marked inhibition of glutamate labeling. Pretreatment with cycloheximide did not affect the content of the phosphate metabolites but slowed down the rate of glutamate labeling (by ≈50%) and of lactate synthesis (by 40%). Table 1 summarizes the rates of glucose consumption, lactate production, and glutamate labeling (at carbon 4) measured under the various conditions used in this study.

DISCUSSION

The ability to monitor cellular metabolism in the NMR spectrometer provided a unique tool for detecting biochemical changes in T47D human breast cancer cells at early times after estrogen administration. The metabolic activities followed in this study included changes in the concentration of phosphate metabolites and in the rate of glucose metabolism through glycolysis and the Krebs cycle.

Within the first 24 hr of estrogen stimulation, the concentration of all of the phosphate metabolites, except that of phosphocholine remained constant within experimental error. On the other hand, the simultaneous measurements of the rate of glucose consumption, lactate production, and glutamate labeling have shown unequivocally an almost immediate enhancement in all three rates up to a factor of 2 at ≈4 hr after estrogen stimulation. These processes are directly associated with energy production and therefore their stimulation should result in a higher ATP content unless there is a concomitant increase in the rate of reactions that utilize ATP. Since ATP remained constant, it can be deduced that following estrogen stimulation the increased energy production balances exactly the increase in energy utilization. This indicates that the energetics of these cells is highly regulated.

To find out whether the mechanism of the observed estrogen-induced changes in glucose metabolism involves RNA and protein synthesis, drugs known to inhibit these processes—actinomycin D at a dose that inhibits mRNA synthesis and cycloheximide, which inhibits protein synthesis—were added either prior to estrogen or together with estrogen. Since the half life of the estrogen receptors in MCF7 human breast cancer cells was found to be of the order of 3–5 hr (14), the pretreatment with the inhibitors could cause a loss of estrogen receptors and therefore inhibit estrogen-induced glucose metabolism indirectly. The simultaneous addition of the inhibitor with estrogen minimized indirect inhibition due to the loss of receptors (14–15). The observed inhibition was therefore most likely due to direct inhibition of synthesis of enzymes involved in glucose metabolism.

It is important to note that in MCF7 cells treated with actinomycin D the estrogen-binding capacity remained high relative to this capacity in estrogen-treated cells (14, 15). This finding was attributed to inhibition of receptor "processing," and therefore such inhibition may provide another indirect mechanism for actinomycin D (but not for cycloheximide) effects on glucose metabolism.

The mechanism by which estrogen induces cell growth and gene expression in breast cancer is not well characterized. It remains a controversial issue whether there is a direct and pivotal role for estrogen in the regulation of breast cancer cell growth (19, 20). The experiments described here show that estrogen stimulation of glucose metabolism requires *de novo* synthesis of proteins and possibly mRNA, thus suggesting that estrogen induction of metabolic activities in breast cancer cells occurs at the translational and probably transcriptional levels. The results therefore support the hypothesis of a direct gene expression mechanism of steroid hormone induction in these cells.

The change in phosphocholine following estrogen administration may reflect an increase in the rate of choline phosphorylation, similar to that found previously in the rat uterus (21). Elevation of the content of phosphocholine was also reported in cultured fibroblasts stimulated by mitogenic factors such as serum, insulin, epidermal growth factor, and others (22). This effect seems to be transient since the phosphocholine concentration in long-term estrogen-treated cells was lower than that in tamoxifen-treated cells (3).

In addition to the specific inhibition of estrogen-induced glucose utilization, cycloheximide and actinomycin D modulated other metabolic processes: cycloheximide caused a decrease in the rate of glycolysis and the Krebs cycle in the presence of either tamoxifen or estrogen. The changes appeared reversible since restoration to initial rates was achieved 1 hr after removing the drug. Actinomycin D, which is a DNA-intercalating chemotherapeutic agent and has a high cytotoxic activity, induced an increase in the concentration of the high-energy phosphates and a decrease in the intracellular P_i concentration and also inhibited glutamate labeling. These changes were irreversible upon switching to actinomycin D-free medium, probably due to the tight DNA intercalation of this drug. Similar changes were observed when the cells were treated with another DNA-intercalating chemotherapeutic drug, adriamycin (M.N., H. Eldar, and H.D., unpublished results). An increase in the content of the high-energy phosphate metabolites was also observed in several NMR studies concerned with the early effects of chemotherapeutic drugs on implanted tumors (23, 24). However, an opposite effect of lowering of ATP was observed when a high dose of actinomycin D (50 μg/ml) was administered to human leukemic leukocytes (25). It is important to note that in the tumors the effects could be attributed to changes in vascularization or in other tissue components, whereas in the perfused cells changes reflect intrinsic modulation in intracellular metabolism.

In summary, the early estrogen induction of the energetics of the cells was exhibited in the stimulation of the energy-producing pathways: glycolysis, which in T47D cells provides most of the energy even under aerobic conditions, and the Krebs cycle. This stimulation did not modulate the concentration of the high-energy phosphates, implying that stimulation of energy-utilizing reactions occurs in parallel to the enhancement in energy production. These changes were suppressed in the presence of actinomycin D and cycloheximide, suggesting that estrogen stimulation of glucose metabolism requires *de novo* synthesis of proteins and possibly of mRNA.

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1. Adams, D. J., Edwards, D. P. & McGuire, W. L. (1983) *Biomembranes* **11**, 389–414.
2. Arafah, B. M., Griffin, P., Gordon, N. H. & Pearson, O. H. (1986) *Cancer Res.* **46**, 3268–3272.
3. Neeman, M. & Degani, H. (1989) *Cancer Res.* **49**, 589–594.
4. Pourreau-Schneider, N., Berthois, Y., Gandilhon, P., Cau, P., Martin, P. M. & Passerel, M. (1986) *Mol. Cell. Endocrinol.* **48**, 77–88.
5. Lima, G. & Shiu, R. P. C. (1985) *Cancer Res.* **45**, 2466–2470.
6. Neeman, M., Rushkin, E., Kadouri, A. & Degani, H. (1987) in *Modern Approaches to Animal Cell Technology*, 8th Meeting of the European Society for Animal Cell Technology, eds. Spier, R. E. & Griffiths, J. B. (Butterworths, London), pp. 328–341.
7. Neeman, M., Rushkin, E., Kadouri, A. & Degani, H. (1988) *Magn. Reson. Med.* **7**, 236–242.
8. Fantini, J., Galons, J. P., Marvaldi, J., Cozzone, P. J. & Canioni, P. (1987) *Int. J. Cancer* **39**, 255–260.
9. Edwards, D. P., Adams, D. J. & McGuire, W. L. (1980) *Biochem. Biophys. Res. Commun.* **93**, 804–812.
10. Vignon, R., Terqui, M., Westley, B., Derocq, D. & Rochefort, H. (1980) *Endocrinology* **106**, 1079–1086.
11. Berthois, Y., Katzenellenbogen, J. A. & Katzenellenbogen, B. S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2496–2500.
12. Osborne, C. K., Boldt, D. H. & Estrada, P. (1984) *Cancer Res.* **44**, 1433–1439.
13. Perry, R. P. & Kelley, D. E. (1970) *J. Cell. Physiol.* **76**, 127–140.
14. Horwitz, K. B. & McGuire, W. L. (1980) *J. Biol. Chem.* **255**, 9699–9705.
15. Strobl, J. S., Kasid, A., Huff, K. K. & Lippman, M. E. (1984) *Endocrinology* **115**, 1116–1124.
16. Huff, K. K. & Lippman, M. E. (1984) *Endocrinology* **114**, 1702–1710.
17. Aitken, S. C., Lippman, M. E., Kasid, A. & Schoenberg, D. R. (1985) *Cancer Res.* **45**, 2608–2615.
18. Neeman, M., Rushkin, E., Kaye, A. M. & Degani, H. (1987) *Biochim. Biophys. Acta* **930**, 179–192.
19. Shafie, S. M. (1980) *Science* **209**, 701–702.
20. Soto, A. M. & Sonnenschein, C. (1984) *Biochem. Biophys. Res. Commun.* **122**, 1097–1103.
21. Spooner, P. M. & Gorski, J. (1972) *Endocrinology* **91**, 1273–1283.
22. Warden, C. H. & Friedkin, M. (1984) *Biochim. Biophys. Acta* **792**, 270–280.
23. Steen, R. G., Tamargo, R. T., McGovern, K. A., Rajan, S. S., Brem, H., Wehrle, J. P. & Glickson, J. D. (1988) *Cancer Res.* **48**, 676–681.
24. Evelhoch, J. L., Keller, N. A. & Corbett, T. H. (1987) *Cancer Res.* **47**, 3396–3401.
25. Laszlo, J., Miller, D. S., McCarty, K. S. & Hochstein, P. (1966) *Science* **151**, 1007–1010.