0026-895X/91/061029-07$03.00/0

MOLECULAR PHARMACOLOGY, 40:1029–1035

Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on 17β-Estradiol-Induced Glucose Metabolism in MCF-7 Human Breast Cancer Cells: ^{13}C Nuclear Magnetic Resonance Spectroscopy Studies

T. R. NARASIMHAN, S. SAFE, H. J. WILLIAMS, and A. I. SCOTT

Department of Veterinary Physiology and Pharmacology (T.R.N., S.S.) and Department of Chemistry (H.J.W., A.I.S.), Texas A & M University, College Station, Texas 77843

Received January 23, 1991; Accepted September 23, 1991

SUMMARY

The effects of 17β-estradiol, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and their combination on the metabolism of [1-^{13}C] glucose were determined in cell suspensions of wild-type MCF-7 human breast cancer cells, by ^{13}C NMR spectroscopy. Preliminary studies showed that, during the 7-hr duration of the NMR experiment, the cells maintained their viability and their aryl hydrocarbon responsiveness. Lactate was the major glucose metabolite detected in these studies, and the rate of lactate formation in the untreated (control) and 17β-estradiol (10^{-9} M)-treated cells was 60 and 86 fmol/cell/hr, respectively; this represented a 40% increase in lactate formation in the cells treated with 17β-estradiol; comparable results were observed for the percentage of glucose converted into lactate. In contrast, TCDD (10^{-9} M) did not significantly alter the rate of glucose metabolism or lactate formation. Co-treatment of the cells with 17β-estradiol (10^{-9} M) plus TCDD (10^{-8} to 10^{-10} M) showed that TCDD completely inhibited the 17β-estradiol-induced metabolism of [^{13}C] glucose to lactate in MCF-7 cells. In contrast, 2,8-dichlorodibenzo-*p*-dioxin (10^{-8} M), a weak aryl hydrocarbon receptor agonist, did not inhibit estrogen-induced glucose-to-lactate metabolism in MCF-7 cells. In addition, it was shown that TCDD caused a significant decrease in 17β-estradiol-induced lactate formation within 1 hr after treatment, whereas the induction of monooxygenase activity was not observed until 3 hr after exposure of the cells to TCDD. These data indicate that TCDD-induced 17β-estradiol metabolism is not related to the decrease in the rate of conversion of glucose to lactate. These results further define the antiestrogenic responses elicited by TCDD and show that ^{13}C NMR spectroscopy provides a unique method for measuring, in real time, the effects of TCDD on specific metabolic pathways.

Transformed human breast cancer cell lines are extensively used as models for understanding the cellular and molecular mechanisms associated with the proliferation of mammary tumors (reviewed in Refs. 1–5). Several studies have established that a variety of hormones can influence the growth of both breast cancer cell lines *in vitro* and mammary tumors. 17β-Estradiol and related estrogens stimulate the proliferation of diverse estrogen-responsive cell lines, which typically express the estrogen receptor protein. The mechanisms of estrogen-induced mammary tumor cell growth are complex and involve the direct effects of estrogen on target cells and also the effects of the estrogen-induced secretion of proteins that may exhibit autocrine or paracrine growth factor activity (reviewed in Refs. 1–5).

Several classes of compounds, including progestins, antiprogestins, androgens, and other steroidal and nonsteroidal antiestrogens that act through the estrogen receptor, inhibit the growth of breast cancer cells or are used for endocrine treatment of breast cancer in women (6–8). Tamoxifen, a clinically used nonsteroidal antiestrogen, binds with moderately high affinity to the estrogen receptor but exhibits only minimal activity as an estrogen agonist in estrogen-responsive human breast cancer cells (6, 9, 10). Moreover, co-treatment of estrogen-responsive breast cancer cell lines with both 17β-estradiol and tamoxifen can result in significant inhibition of a number of estrogen-induced responses, including cell proliferation and the secretion of several proteins including 52- and 160-kDa proteins (6, 9–15).

Recent studies have also demonstrated that TCDD also exhibits a broad spectrum of antiestrogenic activities in estrogen-responsive MCF-7 cells. For example, TCDD down-regulates the nuclear estrogen receptor and inhibits 17β-estradiol-induced cell proliferation, postconfluent focus production, tissue plasminogen activator activity, and the secretion of the 34-, 52-, and 160-kDa proteins (16–19). Mechanistic studies suggest that the antiestrogenic responses elicited by TCDD occur via

This work was supported by the National Institutes of Health (Grant ESO-04176), the Texas Agricultural Experiment Station, The Robert A. Welch Foundation, and The Davidson Endowment. S.S. is a Burroughs Wellcome Toxicology Scholar.

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Ah, aryl hydrocarbon; DCDD, 2,8-dichlorodibenzo-*p*-dioxin; EROD, ethoxyresorufin *O*-deethylase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

initial binding to the Ah receptor and not to the estrogen receptor, because TCDD does not bind to this steroid hormone receptor. The molecular mechanisms associated with this response have not been delineated, but it has been suggested that these effects may be associated with TCDD-induced metabolism of 17β-estradiol (20, 21) or the induction of other gene products that inhibit estrogen-induced gene expression (18, 19).

NMR spectroscopy has been adapted to study the effects of estrogens and other mitogens and growth inhibitors on actively growing breast cancer cells in culture (20–25). Using both ^{31}P and ^{13}C NMR techniques, it was shown that there were significant differences in the concentrations of specific phosphorylated metabolic intermediates and the rate of glucose metabolism between cells treated with 17β-estradiol or tamoxifen. This paper uses ^{13}C NMR techniques to investigate the glycolysis of [^{13}C]glucose in MCF-7 human breast cancer cells treated with 17β-estradiol, TCDD, and related compounds and in cells cotreated with 17β-estradiol plus TCDD. This technique has not been used previously to study the effects of TCDD on fundamental cellular processes, such as glycolysis, using intact cells in which biochemical transformations can be observed without using cellular disruption and fractionation techniques. Moreover, it was also of interest to determine whether there was a parallel between the antiestrogenic responses observed for TCDD (16–19) and the effects of this compound on glycolysis in MCF-7 cells treated with 17β-estradiol.

Materials and Methods

Chemicals and biochemicals. TCDD and DCDD were synthesized in this laboratory to >99% purity, as determined by gas chromatographic and spectroscopic methods. The [^{13}C]glucose was purchased from MSD Isotopes Inc. (Montreal, Canada). All other chemicals and biochemicals were of the highest purity available from commercial sources. MCF-7 human breast cancer cells were purchased from the American Type Culture Collection.

Treatment of cells. MCF-7 cells were grown in minimum essential medium supplemented with 9.5 g/liter HEPES buffer, 2.2 g/liter sodium bicarbonate, 10 mM sodium pyruvate, 6 μg/liter insulin, 10% fetal calf serum, 50 μg/ml gentamicin sulfate, 50 μg/ml amphotericin B, 25 μg/ml penicillin, and 25 μg/ml streptomycin. Stock cultures were maintained in 150-cm^2 tissue culture plates and incubated at 37° in a humidified mixture of 5% CO_2 and 95% air, under atmospheric pressure. After reaching 80–85% confluency, the cells were treated for 12 hr with TCDD (0.1–10 nM), 17β-estradiol (1 nM), DCDD (10 nM), or their combination, using dimethyl sulfoxide (<0.1% of the total volume) as the solvent. At the end of the incubation period with the test compounds, the cells were trypsinized and washed; 4×10^6 cells/3 ml of medium were used for all the NMR studies.

^{13}C NMR apparatus design. In order to maintain viable cells in a uniform medium for these experiments, it was necessary to stir and aerate the culture. These requirements can be a major problem when the cells are contained in an NMR tube located in a superconducting magnet. Contamination by foreign organisms must be avoided and special techniques must be developed to permit stirring and aeration in an NMR sample tube without degradation of magnetic field homogeneity. An air-pumped cell design described by Santos and Turner (25) was used as a starting point for the design of the apparatus used in these studies. In the device, a 4-mm centered hole through a 9-mm-o.d. Teflon vortex plug allowed for the insertion of a 47-mm-long × 4-mm-o.d., 3.1-mm-i.d. glass tube of NMR quality (Fig. 1A). Six 1-mm-diameter holes were arranged symmetrically around the large central hole in the vortex plug, and the device was pressed into a 10-mm-o.d., 9-mm-i.d. NMR tube, until the lower end was within 2 mm of the bottom of the tube. Adjustment of pump position and pump removal after the NMR experiments required the addition of a loop of 5-lb monofilament extending through two adjacent 1-mm holes to a point outside the tube. The monofilament was held in place during experiments by the NMR tube cap. Sufficient liquid was present to rise 5 mm above the top edge of the open central tube for proper pumping action.

A Teflon syringe needle extended from the cap of the NMR tube to a point inside the Teflon plug, and a stream of 95% air/5% carbon dioxide produced small bubbles that pumped the fluid through the cell as they escaped (Fig. 1A). Because the bubbles were above the NMR coil area, little or no change in field homogeneity occurred during this process. However, it was apparent that the cell suspension was constantly circulating throughout the NMR tube and exposed to the 95% air/5% carbon dioxide gas mixture, thus ensuring aeration of the cells. The air-inlet line terminated in a 1-ml plastic syringe, which was attached by its Luer connector to a 0.1-μm disposable plastic syringe filter, which in turn was inserted through a hole in the NMR tube cap and attached to the Teflon tubing (Fig. 1B). Commercial sterile filters and all other parts were autoclaved before use, except the NMR tube cap, which was washed with alcohol before use. The very accurate long-term air-flow needed was provided by a Nupro series S needle valve. With care in handling and utilization of proper sterile techniques, cell culture metabolism studies of several days duration were performed using this system.

^{13}C NMR studies of glucose metabolism in MCF-7 cells. A Bruker WM 30 wide-bore NMR spectrometer with a 10-mm C/H probe was used for all the experiments. MCF-7 cells in culture were normally pretreated with 17β-estradiol, TCDD, DCDD, or their combinations for 12 hr before the NMR experiments. The MCF-7 cells (4×10^6), in 2.7 ml of medium containing the appropriate hormone/chemical and 22 μmol of [^{13}C]glucose in 300 μl of D_2O, were transferred to the NMR apparatus (Fig. 1), for a total volume of 3.0 ml, and aeration was initiated. NMR spectra were recorded in 21-min blocks (1700 scans, 45° ^{13}C pulse, 0.75-sec recycle period, WALTZ decoupling during acquisition) for the duration of the experiment (7 hr). In the time-dependent studies, 11 μmol of [^{13}C]glucose and 1 nM TCDD were added at the beginning of the NMR experiment; the cells were pretreated for 12 hr with 1 nM 17β-estradiol. The cells from the TCDD/17β-estradiol pretreatment studies were collected from the NMR tube after the experiment and stored in liquid nitrogen until used for the determination of enzyme activity. The rate of lactate formation was determined from the terminal 7-hr data. All of the NMR experiments were repeated at least three times, and the results are means ± standard deviations. The differences between treatment groups were determined by Student's t test or analysis of variance.

EROD activity and cell viability. The MCF-7 cells were centrifuged at 1000 × g for 5 min (2°) and either stored for future enzyme assay or resuspended in 200 μl of Tris-sucrose (38 mM Tris buffer, 0.2 M sucrose, pH 8.0). Aliquots of the cell suspension were used for measurement of the EROD activity by the method of Pohl and Fouts (26). Total cell counts and viable cell number were assessed by the trypan blue dye exclusion method, using a hemocytometer. The cell viability and EROD induction data (Table 1) are expressed as means ± standard deviations of at least three separate determinations. The differences between treatment groups were determined by Student's t test or analysis of variance.

Oxygen concentrations in the media. The oxygen concentrations in the cell media were determined by the method described by Robinson and Cooper (27), using a Gilson model oxygraph fitted with a Clark electrode; the oxygraph cell was maintained at a temperature of 37°. The determinations were carried out in triplicate, and the results are expressed as means ± standard deviations.

Results

Most previous NMR studies on actively growing breast cancer cell lines have used cells that were first seeded on agarose-

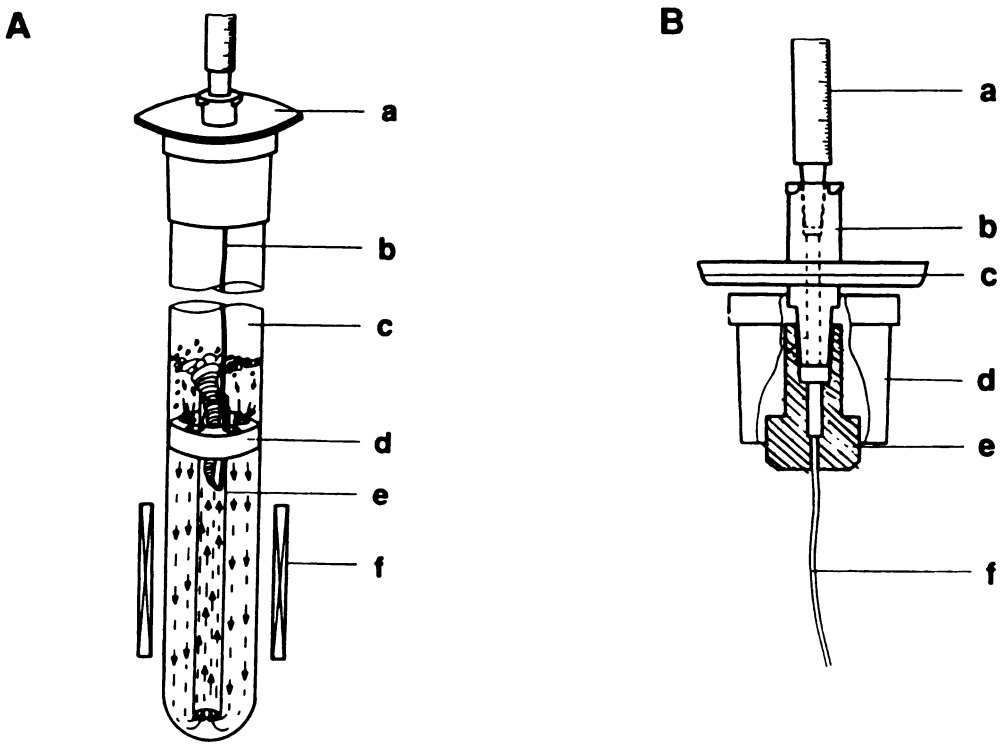

Fig. 1. ^{13}C NMR cell design. The major components illustrated in A outline the configuration of the pump apparatus, which includes the filter (*a*), Teflon tubing (*b*), 10-mm 8-inch NMR tube (*c*), Teflon plug with perforations for liquid movement (*d*), 5-mm inner tubing (*e*), and NMR receiver coils (*f*). B illustrates details of the cap assembly, which include the plastic 1-ml syringe, cut off and attached to the air inlet (*a*), Luer connector (*b*), filter (*c*), NMR tube cap with centered hole for filter outlet (*d*), Luer connector (*e*), and Teflon tubing (*f*).

derived surfaces, followed by perfusion of the growth medium containing ^{13}C-labeled glucose plus specific test chemicals or hormones (20–24). The experiments described in this study used a different procedure, in which cells were suspended in a portion of the apparatus (Fig. 1) between the NMR coils. The system was designed to maximize sterility over long time periods and to provide a homogeneous suspension of cells in which there was minimal disruption of the culture medium in the NMR sample tube. Two studies were carried out to determine the integrity of the cells for the duration of the 7-hr NMR experiment. The results in Table 1 compare the viability of the MCF-7 cells in the various treatment groups both before and after the NMR experiment. The overall percentage of cell viability (based on trypan blue exclusion measurements) was >89% in all the treatment groups, and there were only minimal differences in the viability of cells before and after the NMR experiments. The results in Table 1 summarize the effects of TCDD, 17β-estradiol, and their combination on the induction of the cytochrome P-450-dependent monooxygenase activity EROD, in MCF-7 cells, before and after the NMR experiments. The data show that the EROD inducibility of the cells in the various treatment groups was not significantly altered during the 7-hr duration of the NMR studies. The oxygen tension was determined in the growth medium alone, the medium containing cells, and the medium containing cells 3 and 7 hr after initiation of the NMR experiment, and the values were 224 ± 32, 212 ± 10.5, 215 ± 29, and 264 ± 29 μM, respectively. These data confirm that the cells were sufficiently aerated during the NMR experiment, because the values obtained for samples from the NMR experiment were similar to those observed for samples exposed to the ambient atmosphere.

Preliminary experiments (data not shown) utilized different concentrations of [1-^{13}C]glucose and numbers of cells and, at a [^{13}C]glucose concentration of 22 μmol in 3 ml of medium with 4×10^6 cells, there was a linear rate of glucose utilization and lactate formation over the 7-hr duration of the NMR experiment. The ^{13}C NMR spectra of control and treated cells were similar to those previously reported (20–22) (Fig. 2). The major

TABLE 1
Cell viability and EROD inducibility of MCF-7 cells before and after ^{13}C NMR experiments

Treatment	Before NMR		After NMR	
	Cell viability[a]	EROD activity	Cell viability[a]	EROD activity
	%	*pmol/min/mg of protein*	%	*pmol/min/mg of protein*
Control	97 ± 1.0	2 ± 2	92 ± 0.6	11 ± 1
17β-Estradiol	96 ± 2	1.0 ± 0.5	93 ± 2.5	2.3 ± 0.2
TCDD (10^{-9} M)	95 ± 2	260 ± 35[b]	90 ± 2.0	280 ± 47[b]
17β-Estradiol + TCDD (10^{-8} M)	96 ± 1.5	280 ± 190[b]	89 ± 0.6	410 ± 20[b]
17β-Estradiol + TCDD (10^{-9} M)	95 ± 1.2	180 ± 30[b]	89 ± 1.2	160 ± 49[b]
17β-Estradiol + TCDD (10^{-10} M)	96 ± 1.5	140 ± 24[b]	91 ± 1.0	146 ± 16[b]
17β-Estradiol + DCDD (10^{-8} M)	95 ± 1.3	21 ± 3.1[b]	91 ± 3.1	29 ± 3.5[b]

[a] Cell viability was determined by trypan blue exclusion studies.
[b] Significantly higher EROD activity ($p < 0.01$) than observed in the untreated (control) or 17β-estradiol-treated cells; there were no significant differences ($p < 0.01$) in the EROD activities in the TCDD- or TCDD- plus 17β-estradiol-treated cells.

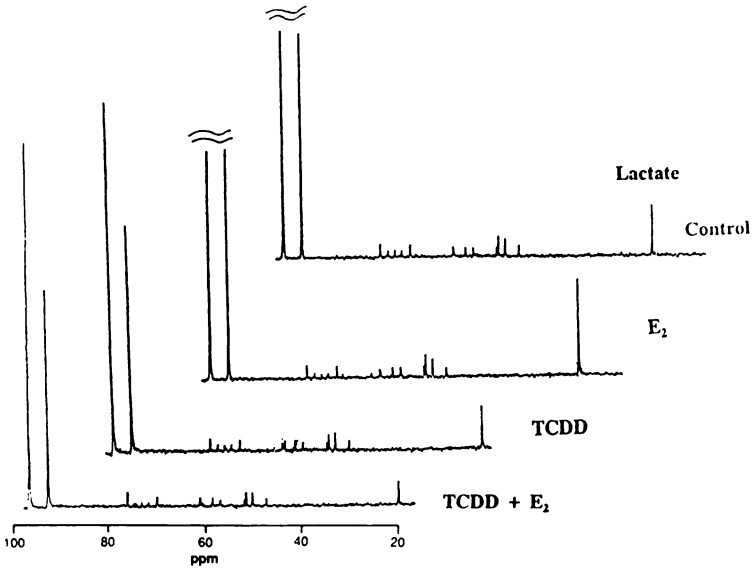

Fig. 2. ^{13}C NMR spectra of MCF-7 cells in suspension. The cells were pretreated with 10^{-9} M 17β-estradiol (E_2), 10^{-9} M TCDD, or TCDD plus 17β-estradiol 12 hr before the NMR experiment. The cells were then suspended in 3 ml of medium containing 22 μmol of [1-^{13}C]glucose, and spectra were recorded as described. The spectra that were observed after 7 hr are illustrated in the figure.

peaks at 92.1 and 95.9 ppm (relative to a dioxane standard at 66.5 ppm) are assigned to C_1 of glucose in the α and β forms, respectively; there was a time-dependent decrease in these peaks, which appeared to be linear over the 7-hr duration of the experiment (Fig. 3). The percentage of [^{13}C]glucose utilized in the formation of lactate in the control and 17β-estradiol- (10^{-9} M), TCDD- (10^{-9} M), and 17β-estradiol- plus TCDD-treated (both 10^{-9} M) MCF-7 cells is illustrated in Fig. 3. 17β-Estradiol significantly increased the rate of glucose-lactate formation in the MCF-7 cells, whereas TCDD did not alter the rate, compared with the control cells. However, it was apparent from the results that TCDD significantly inhibited the 17β-estradiol-induced increase in the conversion of glucose to lactate.

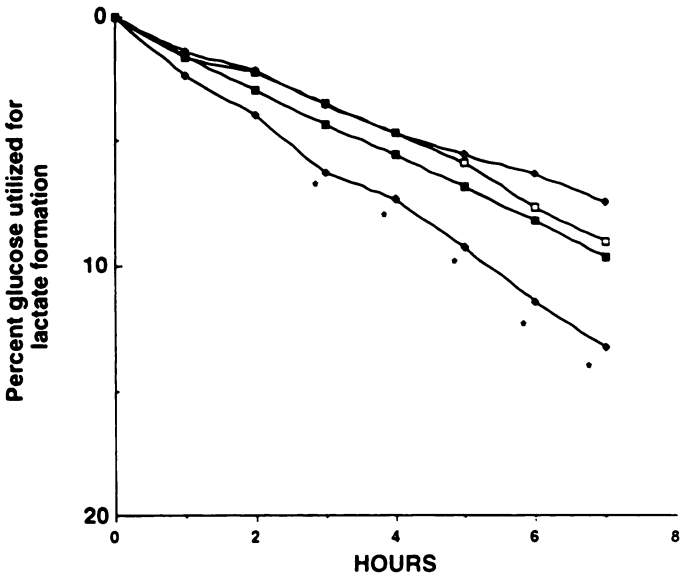

Fig. 3. Percentage of glucose utilized for lactate formation in MCF-7 cells in suspension. The cells were pretreated with solvent (control) (□), 10^{-9} M 17β-estradiol (♦) (*lower curve*), 10^{-9} M TCDD (■), or TCDD plus 17β-estradiol (♦) 12 hr before the NMR experiment, as described. The results are expressed as means ± standard deviations of at least three separate determinations. ★, Significant differences ($p < 0.05$) between the 17β-estradiol- and 17β-estradiol- plus TCDD-treated cells.

The other major peak that was observed in the ^{13}C NMR spectra, at 20.0 ppm, is assigned to C_1 of lactate, and the time-dependent increase in this signal was linear over the 7-hr incubation period (Table 2). The rate of lactate formation in the control and 17β-estradiol- (10^{-9} M), TCDD- (10^{-9} M), and 17β-estradiol- plus TCDD-treated (both 10^{-9} M) MCF-7 cells was 60, 86, 64, and 54 fmol/cell/hr, respectively. 17β-Estradiol caused a 40% increase in the overall rate of lactate formation (compared with the control cells) during the experiment, whereas TCDD did not significantly modulate the rate of lactate formation. In the co-treatment experiments, TCDD significantly inhibited the 17β-estradiol-induced formation of lactate in the MCF-7 cells in suspension.

Several small peaks occurred between 77 and 48 ppm. Most of the peaks were present in the spectra of the medium before the addition of [^{13}C]glucose, and their concentrations remained constant for the duration of the experiment. These include the signals of 75.8, 74.2, 72.8, 71.5, 71.4, 69.7, 68.5, 60.8, 58.2, 56.6, 51.8, 51.4, 50.1, and 47.2 ppm. These peaks were, therefore, attributed to natural abundance signals from medium consitutents. Peaks at 74.2, 60.6, 60.3, and 58.2 ppm increased slightly over a period of 1–2 hr after addition of the [^{13}C]glucose, whereas peaks at 56.6 and 50.1 ppm showed larger increases in intensity during this time period. After 2 hr, a steady state concentration was observed for all these peaks. The peaks near 60 ppm were tentatively attributed to C_1 of fructose phosphates; the positions of other peaks did not match those of the following glycolysis pathway intermediates, Krebs cycle intermediates, and related compounds: glucose-6-phosphate, glyceraldehyde-3-phosphate, dihydroxyacetone, dihydroxyacetone phosphate, glycerine, 1,3-diphosphoglyceric acid, 2-phosphoglyceric acid, 3-phosphoglyceric acid, phosphoenol pyruvic acid, pyruvic acid, aspartic acid, glutamic acid, glutamine, citric acid, and isocitric acid.

The concentration-dependent effects of TCDD on the 17β-estradiol-induced rate of lactate formation and glucose degradation in MCF-7 cells in suspension are summarized in Table 2. The results show that TCDD significantly inhibited 17β-estradiol-induced glycolysis and lactate formation at concentrations as low as 10^{-10} M. In addition, the effects of the weak

TABLE 2

Effects of different treatments on the formation of lactate from 22 μmol of [^{13}C]glucose in MCF-7 human breast cancer cells, determined using ^{13}C NMR

Treatment	Lactate formation at hourly intervals						
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr
	nmol/4 × 10^6 cells						
Control	352 ± 114	474 ± 169	723 ± 132	932 ± 152	1142 ± 170	1446 ± 266	1680 ± 146
17β-Estradiol	519 ± 62[a]	837 ± 86[a]	1285 ± 175[a]	1461 ± 191[a]	1790 ± 177[a]	2154 ± 302[a]	2405 ± 250[a]
TCDD (10^{-9} M)	351 ± 164	623 ± 215	897 ± 270	1111 ± 365	1334 ± 377	1541 ± 428	1793 ± 435
17β-Estradiol + TCDD (10^{-8} M)	385 ± 65[b]	559 ± 46[b]	872 ± 61[b]	1047 ± 42[b]	1249 ± 61[b]	1486 ± 183[b]	1696 ± 266[b]
17β-Estradiol + TCDD (10^{-9} M)	299 ± 76[b]	476 ± 101[b]	762 ± 31[b]	975 ± 114[b]	1138 ± 151[b]	1276 ± 173[b]	1502 ± 237[b]
17β-Estradiol + TCDD (10^{-10} M)	334 ± 104[b]	555 ± 88[b]	754 ± 229[b]	1016 ± 255[b]	1207 ± 264[b]	1432 ± 331[b]	1626 ± 428[b]
17β-Estradiol + DCDD (10^{-8} M)	420 ± 59	731 ± 162	1119 ± 109	1414 ± 109	1761 ± 200	1872 ± 369	2273 ± 538

[a] Significantly different ($p < 0.05$) from the control cells.
[b] Significantly different ($p < 0.05$) from the 17β-estradiol-treated cells.

Ah receptor agonist DCDD on 17β-estradiol-induced glycolysis were also determined at a concentration of 10^{-8} M. The rates of lactate formation in the 17β-estradiol- and 17β-estradiol-plus DCDD-treated cells were 86 and 81 fmol/cell/hr, respectively, and these values were not significantly different from each other.

In the time-dependent studies, 1 nM TCDD and 11 μmol of [^{13}C]glucose were added at the beginning of the NMR experiment, and the effects of TCDD on 17β-estradiol-induced lactate formation were measured by ^{13}C NMR. Within 1 hr after the addition of TCDD, significant inhibition of lactate formation was observed (Table 3), and this persisted for 7 hr. In contrast, significant induction of EROD activity was only observed 3 hr after the addition of TCDD.

Discussion

High-field NMR spectroscopy has been widely used as a tool for understanding and probing mechanisms of diverse biochemical processes (28–30). One of the important advantages of the NMR method is the capability of measuring metabolic intermediates in real time, without disrupting the cells of interest. This technique has been utilized to investigate the effects of growth conditions and growth modulators on rates of metabolic pathways in human breast cancer cell lines (20–24). Most of these studies have utilized a cell perfusion system in which the transformed cells were seeded on agarose-derived matrices (e.g., beads or threads). In the present study, a modified air-pumped cell design was adopted to minimize contamination and maintain a homogeneous suspension of cells for the duration of the experiments (Fig. 1). A stream of 95% air/5% carbon dioxide entered the system above the NMR coil area, with little or no degradation of field homogeneity during the experiment. The cells within the NMR coil area were maintained in uniform suspension. The integrity of the MCF-7 cells in the experiment was determined by comparing some of the properties of cells from the same experiment before and after the NMR experiments. The results summarized in Table 1 illustrate that, for several different treatment groups, the viability of cells before and after the NMR experiments was >89% over the 7-hr duration of the study, with only minor differences in cell viability within the treatment groups. Previous reports have demonstrated that MCF-7 cells express the Ah receptor (18, 31, 32) and TCDD induces *CYPIA1* gene expression in this cell line. This induced response can be monitored by determining EROD activity in the cells, and the results summarized in Table 1 indicate that the EROD inducibility of cells was essentially unchanged before and after the NMR experiment. Thus, the cell suspensions that were used in this study maintained their viability and Ah responsiveness during the course of the NMR experiments. Jaroszewski and co-workers (23) have investigated the effects of gossypol and rhodamine-123 on glucose metabolism and the levels of phosphorylated metabolic intermediates in MCF-7 cells, using ^{13}C and ^{31}P NMR spectroscopy. In their studies, it was reported that gossypol altered cellular membranes and surface proteins, and this resulted in significant loss of attachment of the cells to the gel matrix. Their NMR measurements on MCF-7 cells were obtained by first treating the cells with the appropriate chemical for specific times and then measuring the metabolite levels in aerated cell suspensions within 1.5 hr after harvest. The results reported in the present study suggest that, by using the cell design shown in Fig. 1, on-line NMR measurements of metabolites can be determined using viable and responsive MCF-7 cells in suspension.

TABLE 3

Inhibition of 17β-estradiol-induced metabolism of [^{13}C]glucose in MCF-7 cells by TCDD: time-dependent effects

Cells were treated as indicated, and lactate formation and EROD activity were measured.

Time	17β-Estradiol (1 nM)	17β-Estradiol (1 nM) plus TCDD (1 nM)	
	Lactate	Lactate	EROD
hr	*nmol/4 × 10^6 cells*	*nmol/4 × 10^6 cells*	*pmol/min/mg*
1	214 ± 21	123 ± 30[a]	Not detected
2	252 ± 15	147 ± 14[b]	Not detected
3	358 ± 18	182 ± 48[b]	12.4 ± 1.5
4	463 ± 24	183 ± 41[b]	
5	543 ± 17	221 ± 69[b]	32.3 ± 3.5
6	663 ± 20	270 ± 89[b]	
7	838 ± 24	304 ± 27[b]	45.1 ± 3.7

[a] In this experiment, the cells were pretreated with 1 nM 17β-estradiol for 12 hr before the addition of 11 μmol of [^{13}C]glucose and 1 nM TCDD (time 0).
[b] Significantly different ($p < 0.05$) from the 17β-estradiol-treated cells.

formation (Table 3), the concentration of [^{13}C]glucose was 11 μmol/3 ml. In this study, lactate was the major metabolite derived from [1-^{13}C]glucose, and glutamate and alanine were not observed in the ^{13}C NMR spectra in any of the treatment groups. This result was similar to the observations reported by Lyon *et al.* (20). The effects of 17β-estradiol on the percentage of conversion of glucose to lactate (Fig. 3) and formation of lactate (Table 2) were monitored by periodic ^{13}C NMR scans over a period of 7 hr. The formation of lactate was linear over the duration of the 7-hr experiment for both the control and 17β-estradiol-treated cells, and the average rates of lactate formation were 60 and 86 fmol/cell/hr, respectively. Thus, there was a >40% increase in the rate of lactate formation in the 17β-estradiol-treated cells, compared with the control. Previous studies by Neeman and Degani (22) did not report the control versus 17β-estradiol-induced rates of lactate formation in MCF-7 cells; however, in the hormone-treated cells the rate was 560 ± 60 fmol/cell/hr. The differences between the rates of lactate formation (i.e., 86 versus 560 fmol/cell/hr) may be due to several factors, including the number of cells used (4×10^6 versus 6×10^7), the volume of medium (3 ml versus 50 ml), the concentration of glucose (22 μmol in 3 ml versus 50 mg or 276 μmol in 50 ml), the duration of the experiments (7 hr versus 4 hr), and the use of suspended versus adhered cells, respectively. In the study by Lyon *et al.* (20), the [^{13}C]glucose/number of cells ratio was 5 mM/5×10^8 (in 20 ml); in both wild-type and Adriamycin-resistant MCF-7 cells, steady state concentrations of lactate were observed after 100 and 200 min, respectively, and the estimated rates of lactate formation were <6 and <2.5 fmol/cell/hr, respectively. Thus, the rate data obtained in the present study (Table 2) were between the values previously reported in the literature (21, 22).

Like the nonsteroidal antiestrogen tamoxifen (22), TCDD exhibits a broad spectrum of antiestrogenic responses in estrogen-responsive human breast cancer cell lines. Neeman and Degani (22) reported that in the presence of tamoxifen the rates of [^{13}C]glucose metabolism, in wild-type MCF-7 cells, to give lactate and glutamate were 250 and 2.9 fmol/cell/hr, respectively, and these rates were approximately 50% lower than those observed in cells treated with 17β-estradiol alone. The rate of formation of these metabolites in control cells was not reported. In the present study, the effects of a wide range of concentrations of TCDD on [^{13}C]glucose metabolism were investigated (Table 2), and the results indicate that, at concentrations of $\leq 10^{-8}$ M, the rates of lactate formation in the TCDD-treated and control cells were not significantly different but were lower than those observed in cells treated with 10^{-9} M 17β-estradiol. Thus, although both TCDD and tamoxifen inhibit the growth of MCF-7 cells, TCDD does not alter the rate of glycolysis in these cells.

The interactions of 17β-estradiol and different concentrations of TCDD on glucose metabolism were also investigated by ^{13}C NMR. The results (Table 2; Fig. 3) clearly show that 10^{-8} to 10^{-10} M concentrations of TCDD significantly inhibited the 17β-estradiol-induced rate of lactate formation. In contrast, co-treatment of the MCF-7 cells with 10^{-8} M DCDD, a weak Ah receptor agonist (33), did not significantly inhibit 17β-estradiol-induced [^{13}C]glucose metabolism in MCF-7 cells. Previous studies did not report whether tamoxifen inhibited 17β-estradiol-induced glucose metabolism in MCF-7 cells; however, it is apparent from the results in Table 2 that TCDD significantly inhibited this estrogen-induced response.

The results from this study and others clearly support the role of the Ah receptor in mediating the antiestrogenic responses to TCDD and related compounds (16–19). It has been proposed that these effects are the result of TCDD-induced 17β-estradiol metabolism (134, 135) or the induction by TCDD of gene products that influence estrogen-induced gene transcription (18, 19). The effect of TCDD-induced 17β-estradiol metabolism on the rate of lactate formation was investigated by adding TCDD and [^{13}C]glucose at the same time (time 0) and measuring the time-dependent formation of lactate in cells treated with 17β-estradiol or 17β-estradiol plus TCDD (Table 3). TCDD significantly inhibited the 17β-estradiol-induced formation of lactate at all time points from 1 to 7 hr after the addition of TCDD. In contrast, TCDD significantly induced EROD activity, a marker for the induction of cytochrome P-4501A1 (34, 35), only after 3 hr. These data suggest that the TCDD-mediated inhibition of 17β-estradiol-induced lactate formation in MCF-7 cells is not dependent on the concomitant oxidative metabolism of the hormone.

Future studies in this laboratory will utilize the ^{13}C NMR technique to further investigate the mechanism or mechanisms associated with TCDD-induced antiestrogenic responses and the parallel inhibitory effects of TCDD on growth factor-induced proliferation of human breast cancer cells.

References

1. Dickson, R. B., and M. E. Lippman. Estrogenic regulation of growth and polypeptide growth-factor secretion in human breast carcinoma. *Endocr. Rev.* **8**:29–43 (1987).
2. Dickson, R. B., and M. E. Lippman. Hormonal control of human breast cancer cells. *Cancer Surv.* **5**:617–624 (1986).
3. Lippman, M. E., and R. B. Dickson. Mechanisms of growth control in normal and malignant breast epithelium. *Recent Prog. Horm. Res.* **45**:383–440 (1989).
4. Lippman, M. E., and R. B. Dickson. Mechanisms of normal and malignant breast epithelial growth regulation. *J. Steroid Biochem.* **34**:107–121 (1989).
5. Osborne, C. K., and C. L. Arteaga. Autocrine and paracrine growth regulation of breast cancer: clinical implications. *Breast Cancer Res. Treat.* **15**:3–11 (1990).
6. Lerner, L. J., and V. C. Jordan. Development of antiestrogens and their use in breast cancer: Eighth Cain Memorial Award Lecture. *Cancer Res.* **50**:4177–4189 (1990).
7. Santen, R. J., A. Manni, H. Harvey, and C. Redmond. Endocrine treatment of breast cancer in women. *Endocr. Rev.* **11**:221–265 (1990).
8. Chamness, G. C. Progestin action and progesterone receptors in breast cancer. *Cancer Res.* **49**:7176–7179 (1989).
9. Miller, M. A., Y. Y. Sheen, A. Mullick, and B. S. Katzenellenbogen. Antiestrogen binding to estrogen receptors and additional antiestrogen binding sites in human breast cancer cells, in *Estrogen/Antiestrogen Action and Breast Cancer Therapy* (C. V. Jordan, ed.). The University of Wisconsin Press, Madison, WI, 127–148 (1986).
10. Furr, B. J. A., and V. C. Jordan. The pharmacology and clinical uses of tamoxifen. *Pharmacol. Ther.* **25**:127–205 (1984).
11. Morisset, M., F. Capony, and H. Rochefort. Processing and estrogen regulation of the 52-kDa protein inside MCF-7 breast cancer cells. *Endocrinology* **119**:2773–2782 (1986).
12. Rochefort, H., F. Vignon, S. Bardon, F. May, and B. Westley. Different efficacy of antiestrogens on estrogen-regulated proteins in human breast cancer cell lines, in *Estrogen/Antiestrogen Action and Breast Cancer Therapy* (C. V. Jordan, ed.). The University of Wisconsin Press, Madison, WI, 249–263 (1986).
13. Aitken, S. C., and M. E. Lippman. Effect of estrogens and antiestrogens on growth-regulatory enzymes in human breast cancer-cells in tissue culture. *Cancer Res.* **45**:1611–1620 (1985).
14. Westley, B., F. E. B. May, M. C. Brown, A. Krust, P. Chambon, M. E. Lippman, and H. Rochefort. Effects of antiestrogens on the estrogen-regulated PS2 RNA and the 52-kDa and 160-kDa proteins in MCF-7 cells and two tamoxifen resistant sublines. *J. Biol. Chem.* **259**:10030–10035 (1984).
15. Thompson, E. W., R. Reich, T. B. Shima, A. Albini, J. Graf, G. R. Martin, R. B. Dickson, and M. E. Lippman. Differential regulation of growth and invasiveness of MCF-7 breast cancer cells by antiestrogens. *Cancer Res.* **48**:6764–6768 (1988).
16. Gierthy, J. F., and D. W. Lincoln. Inhibition of postconfluent focus produc-

tion in cultures of MCF-7 breast cancer cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Breast Cancer Res. Treat.* **12**:227–233 (1988).
17. Gierthy, J. F., D. W. Lincoln, M. B. Gillespie, J. I. Seeger, H. L. Martinez, H. W. Dickerman, and S. A. Kumar. Suppression of estrogen-regulated extracellular plasminogen activator activity of MCF-7 cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Cancer Res.* **47**:6198–6203 (1987).
18. Harris, M., T. Zacharewski, and S. Safe. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds on the occupied nuclear estrogen receptor in MCF-7 human breast cancer cell. *Cancer Res.* **50**:3579–3584 (1990).
19. Biegel, L., and S. Safe. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on cell growth and the secretion of the estrogen-induced 34-, 52-, and 160-kDa proteins in human breast cancer cells. *J. Steroid Biochem.* **37**:725–732 (1990).
20. Lyon, R. C., J. S. Cohen, P. J. Faustino, F. Megnin, and C. E. Myers. Glucose metabolism in drug-sensitive and drug-resistant human breast cancer cells monitored by magnetic resonance spectroscopy. *Cancer Res.* **48**:870–877 (1988).
21. Neeman, M., and H. Degani. 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. *Proc. Natl. Acad. Sci. USA* **86**:5585–5589 (1989).
22. Neeman, M., and H. Degani. Metabolic studies of estrogen- and tamoxifen-treated human breast cancer cells by nuclear magnetic resonance spectroscopy. *Cancer Res.* **49**:589–594 (1989).
23. Jaroszewski, J. W., O. Kaplan, and J. S. Cohen. Action of gossypol and rhodamine 123 on wild-type and multidrug-resistant MCF-7 human breast cancer cells: ^{31}P nuclear magnetic resonance and toxicity studies. *Cancer Res.* **50**:6936–6943 (1990).
24. Kaplan, O., J. W. Jaroszewski, P. J. Faustino, G. Zugmaier, B. W. Ennis, M. Lippman, and J. S. Cohen. Toxicity and effects of epidermal growth factor on glucose metabolism of MDA-468 human breast cancer cells. *J. Biol. Chem.* **265**:13641–13649 (1990).
25. Santos, H., and D. L. Turner. Characterization of the improved sensitivity obtained using a flow method for oxygenating in mixing cell suspensions in NMR. *J. Magn. Res.* **68**:345–349 (1986).
26. Pohl, R. J., and J. R. Fouts. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal. Biochem.* **107**:150–155 (1980).
27. Robinson, J., and J. M. Cooper. Method of determining oxygen concentrations in biological media, suitable for calibration of the oxygen electrode. *Anal. Biochem.* **33**:390–399 (1970).
28. Lundberg, P., E. Harmsen, C. Ho, and H. J. Vobel. Nuclear magnetic resonance studies of cellular metabolism. *Anal. Biochem.* **191**:193–222 (1990).
29. Baxter, R. L., N. E. Mackenzie, and A. I. Scott. CMR as a probe for metabolic pathways *in vivo*, in *Biological Magnetic Resonance* (L. J. Berliner and J. Reuben, eds.), Vol. 5. Plenum Publishing Corp., New York, 1–18 (1983).
30. Scott, A. I. Enzyme mechanisms studied by high-field NMR *in vitro* and *in vivo*, in *Chemistry of Texas: 30th Year of Welch Foundation* (N. Dittrich, ed.). Robert A. Welch Foundation, Houston, Texas, 237–282 (1984).
31. Vickers, P. J., M. J. Dufresne, and K. H. Cowan. Relation between cytochrome P454IAI expression and estrogen receptor content of human breast cancer cells. *Mol. Endocrinol.* **3**:157–164 (1989).
32. Harris, M., J. Piskorska-Pliszczynska, T. Zacharewski, M. Romkes, and S. Safe. Structure-dependent induction of aryl hydrocarbon hydroxylase in human breast cancer cell lines and characterization of the Ah receptor. *Cancer Res.* **49**:4531–4535 (1989).
33. Safe, S. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol.* **26**:371–399 (1986).
34. Gierthy, J. F., D. W. Lincoln, S. J. Kampcik, H. W. Dickerman, H. L. Bradlow, T. Niwa, and G. E. Swaneck. Enhancement of 2- and 16α-estradiol hydroxylation in MCF-7 human breast cancer cells in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biochem. Biophys. Res. Commun.* **157**:515–520 (1988).
35. Spink, D. C., D. W. Lincoln, H. W. Dickerman, and J. F. Gierthy. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes an extensive alteration of 17β-estradiol metabolism in human breast cancer cells. *Proc. Natl. Acad. Sci. USA* **87**:6917–6921 (1990).

Send reprint requests to: S. Safe, Department of Veterinary Physiology and Pharmacology, Texas Veterinary Medical Center, Texas A & M University, College Station, TX 77843-4466.