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ESTROGEN SYNTHETASE STIMULATION BY HEMIN IN HUMAN CHORIOCARCINOMA CELL CULTURE

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Summary: The ability of hemin to stimulate estrogen synthetase (aromatase) in cultured human trophoblast cells and in cellular homogenates was investigated and compared with aromatase stimulation by dibutyryl cAMP [$(Bu)_2$cAMP]. Cells grown with hemin for 24 h, or homogenates incubated for 45 min with hemin, showed maximal aromatase stimulation (150 to 200% of activities in the absence of hemin) at 25 μM and 0.1 μM, respectively. Aromatase stimulation in culture by 25 μM hemin was observed within 4 h after hemin addition, while $(Bu)_2$cAMP required more than 6 h. Intracellular heme and porphyrin levels were higher (160 to 185%) in 96 h $(Bu)_2$cAMP-grown cells than control cells.

Regulation of the enzyme which makes estrogens from androgens, estrogen synthetase (aromatase), plays an important role in many physiological processes, such as reproduction and fetal development, and is of clinical interest in several pathological processes, such as estrogen-dependent carcinomas. There are many basic and clinical studies of the pharmacological inhibition (1,2) of this cytochrome P-450 enzyme system (3,4), but relatively few studies of the mechanism of its physiological regulation.

In one model system, human choriocarcinoma cells in culture, $(Bu)_2$cAMP plus theophylline stimulate aromatase activity eight-to-ten-fold over 96 h by a process requiring RNA and protein synthesis (5). Cytochrome P-450 appears to be the regulatory component of aromatase in these cells (5,6). We report

Abbreviations used: $(Bu)_2$cAMP - dibutyryl cAMP, dbT - 1 mM $(Bu)_2$cAMP plus 1 mM theophylline.

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here the ability of hemin to rapidly and significantly stimulate aromatase in these cells. This is the first report of the ability of hemin to stimulate a steroidogenic cytochrome P-450 enzyme in cells in culture as well as in cellular homogenates.

MATERIALS AND METHODS

Materials: The aromatase substrate, [1β-^{3}H, 4-^{14}C] androstenedione (64.4 Ci ^{3}H, 1.7 Ci ^{14}C per mol), was prepared using the drastic alkali treatment (7) on a mixture of [1,2-^{3}H] androstenedione (41 Ci/mmol from NEN) and [4-^{14}C] androstenedione (57.4 Ci/mol from NEN). The distribution of ^{3}H is 77% in the 1β position (by nmr from NEN).

Hemin (Type I, bovine), protoporphyrin IX, oxalic acid, non-radioactive steroids, cytochrome c and NADPH were purchased from Sigma Chemical Co. Liquiscint scintillation cocktail came from National Diagnostics. GIBCO supplied the cell culture media and serum.

Methods:

Cell Culture: Human choriocarcinoma cells (JAr line) were maintained in continuous culture with growth medium changed daily, and grown in the absence or presence of dbT as previously described (8). The appropriate amount of hemin (dissolved under reduced light conditions in 0.1 M NaOH, brought to approximate pH 8.5 with 0.1 N HCl and further diluted with culture medium to the proper concentration) was maintained in the cell culture medium in the dark for the time periods indicated. At termination, the attached cells were washed twice using serum-free culture medium containing the appropriate amount of hemin, removed from the flask and collected by centrifugation as described (9).

Enzyme and Protein Assays: Frozen cell pellets were thawed and homogenized in GP buffer (5). NADPH-cytochrome c reductase activity (5) and protein content (10) were measured as described.

The aromatase assay, performed in duplicate or triplicate, was based on the transfer of ^{3}H from the substrate to $^{3}H_2O$ concomitant with estrogen production (4), using substrate (10^{5} dpm ^{3}H, 200 ng androstenedione), 0.5 mg NADPH and homogenized cells (0.8 to 2 mg protein) in a total volume of 1 ml. Control experiments verified substrate saturation and linearity of the assay. The assay was conducted and $^{3}H_2O$ quantitated as described (9). We verified in selected samples that the apparent activation of aromatase by hemin using the $^{3}H_2O$ assay is correlated with an increase in radiolabeled estrogen production (procedure as in (11)).

Heme/Porphyrin Assay: Cellular heme and porphyrin content were measured by the method of Granick et al.(12) and Sassa et al. (13), modified as described here, after washing the cells twice in 0.15 M NaCl. The cell pellet was homogenized in 0.067 M phosphate buffer, pH 7.4, and aliquots mixed with 1 ml oxalic acid (saturated solution at 21°C) for heme measurement or extracted with 1 ml of PM [mixture of 1 N perchloric acid-methanol (1:1, v/v)] for porphyrin measurement. For the heme assay, samples were heated at 100°C for 30 min, 0.5 ml of PM added after cooling to room temperature, and the 1,000 xg-20 min supernatant assayed. Fluorescence was measured in an Aminco-Bowman spectrofluorometer, with a red-insensitive photomultiplier tube (1P21, S4 spectral response, 0.2 mm slit), at 406 nm excitation (slit-22nm) and emissions (slit-11 nm) of 577-578 nm (minimum) and 596 nm (maximum). Control experiments showed that the

fluorescence was linearly related to the amount of protein in the assay over the range utilized.

Statistical Analysis: All experiments described here were performed at least twice. Except where otherwise noted, all assays were in duplicate or triplicate and the results reported as mean±S.D. Statistical comparisons were made using Student's t-test.

RESULTS AND DISCUSSION

Various concentrations of hemin were incubated for 24 h with control and dbT-grown choriocarcinoma cells in culture and specific activities of aromatase and NADPH-cytochrome c reductase were determined (Fig. 1). The optimal hemin concentration for the specific and significant stimulation of aromatase ($p < 0.005$ relative to aromatase in the absence of hemin) was 25 μM for control cells and about 10 μM for dbT-grown cells. This optimal hemin concentration failed to affect the secretion of human chorionic gonadotropin, glycoprotein hormone α-subunit or progesterone from control cells (R.H., unpublished work). As reported for mouse Friend virus-transformed erythroleukemia cells in culture (14), hemin concentrations above 100 μM was toxic to these cells. These data

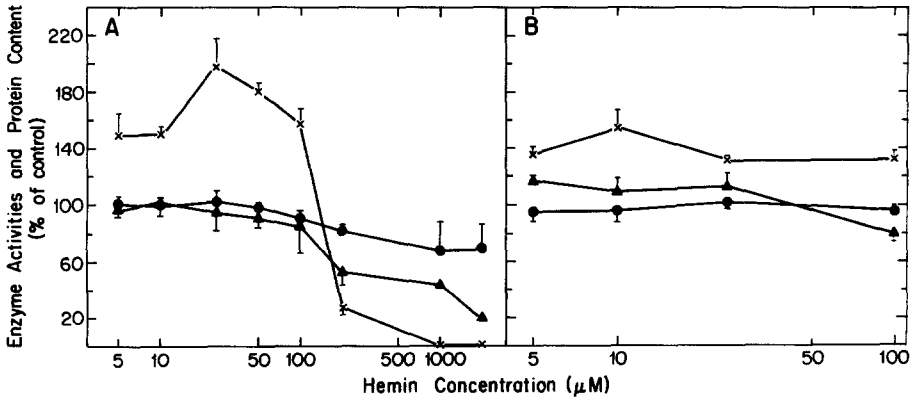

Fig. 1. Aromatase stimulation by hemin in cultured cells. (A) Choriocarcinoma cells were grown with medium containing hemin as indicated. (B) Same as (A) except dbT was included in the medium. The 100% (hemin-free control) values for aromatase (X; pmol/min/mg protein), NADPH-cytochrome c reductase (▲; nmol/min/mg protein), and protein (●; mg/flask) were for (A) 0.23, 2.58 and 5.5, respectively, and for (B) 0.95, 2.55 and 5.5, respectively. These data are accumulated from and representative of five separate determination for control cells and two for dbT-grown cells.

show for the first time, to the best of our knowledge, stimulation by hemin of a cytochrome P-450 enzyme in cultured cells and of a steroidogenic cytochrome P-450 enzyme.

The results in Fig. 1 do not permit identification of the mechanism of heme action. Since previous studies of the stimulation of cytochrome P-450-dependent drug hydroxylation, in hepatic tissues following treatment with agents that promote the formation of apocytochrome P-450 (15) or in untreated non-hepatic tissues (16), were performed in tissue homogenates and/or subcellular fractions, the mechanism of hemin activation is generally regarded to be *via* insertion into the apocytochrome P-450 (17,18). We tested this mechanism of hemin activation by incubating various hemin concentrations with homogenized cells *in vitro*. The results in Fig. 2 show that a similar extent of aromatase activation as that seen with cultured cells occurs at 0.1 μM hemin. Both sets of data taken together suggest that hemin activates aromatase by an insertion mechanism. Furthermore, the extent of activation (150 to 200% of values in the absence of hemin) suggests that about 30 to 50% of the aromatase cytochrome P-450 in these cells is in the apocytochrome form.

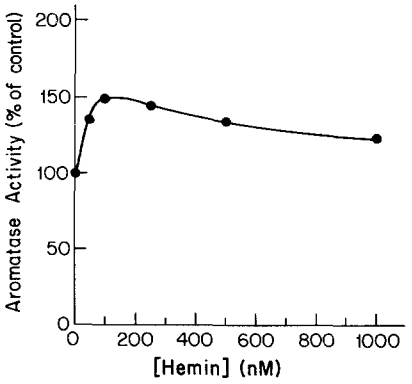

Fig. 2. Aromatase stimulation by hemin in cellular homogenates. Control choriocarcinoma cells were homogenized and incubated with hemin as indicated and 1.5 mM EDTA for 45 min at 37°C in flasks protected from light. The aromatase assay was initiated by adding substrate and NADPH followed by incubation at 37°C for 32 min. The control (100%) aromatase activity was 0.055 pmol/min/mg protein.

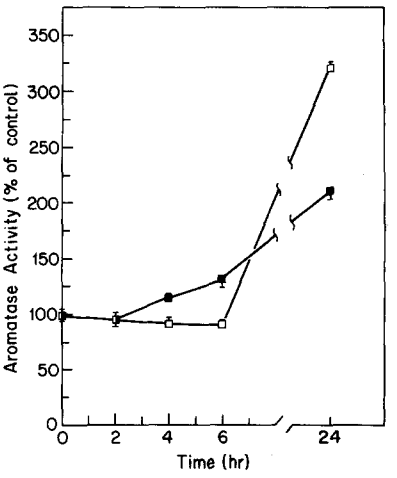

Fig. 3. Time course of hemin - and dbT- stimulation of aromatase in culture. Cells were grown in medium alone (control), medium with 25 μM hemin (■), or medium with dbT (□). Aromatase specific activities in hemin - and dbT - containing medium were divided by the specific activity of control cells at each time point. The average NADPH-cytochrome c reductase activity or protein content throughout the 24 h were not significantly different in control or treated cells. The control aromatase activities (pmol/min/mg protein) at zero time averaged 0.25 ± 0.012 (n=10), increasing to 0.46 ± 0.006 (n=4) at 6 h before declining to 0.3±0.005 (n=4) at 24 h.

To determine if heme plays a role in the well-documented stimulation of aromatase by dbT, we compared the time course of aromatase stimulation by 25 μM hemin and by dbT in cultured cells (Fig. 3). At 4 and 6 h, aromatase activity in hemin-grown cells, but not in dbT-grown cells, was significantly higher than the activity in control cells ($p<0.005$), reaching a maximum value from 24 to 48 h. By 24 h the aromatase activity in dbT-grown cells exceeded the value in control and 24 μM hemin-grown cells.

Furthermore, we sought to determine if intracellular heme and porphyrin levels are responsive to dbT by comparing these levels in cells grown for various periods of time without (control) or with dbT. The data in Table I suggest that only after 96 h growth with dbT, when aromatase activity is stimulated by 8-to 10-fold (5), were higher levels of heme and porphyrin observed in dbT-grown cells relative to control cells.

Table 1. Porphyrin and heme content of choriocarcinoma cells

	time	Porphyrin		Heme	
		control	dbT-grown	control	dbT-grown
		pmol/mg prot.		pmol/mg prot.	
Experiment I	96 h	0.51 ±0.15	0.94 ± 0.42	8.15 ± 2.28	13.45± 1.23*
Experiment II	48 h	1.55 ±0.31	1.25 ± 0.098	11.6 ± 0.71	13.3± 0.47*
	72 h	0.92 ±0.07	1.2 ± 0.063*	11.3 ± 1.35	11.9± 0.96
	96 h	0.72 ±0.1	1.34 ± 0.11*	8.43 ± 0.54	13.3± 0.77*

Cells were grown in medium alone or with dbT for the indicated time, and then washed and collected as described. Aliquots of homogenized cells were assayed for heme and porphyrin content and protein concentration. Porphyrin was undetectable in both growth medium and undiluted serum. Heme levels was 1.57 μM in 10% serum. The total heme content of the growth medium per flask divided by the total cellular protein attached to the flask is about 2.4 nmol heme/mg protein.

* $p < 0.005$ relative to control cells at same time.

Provided that aromatase cytochrome P-450 levels are less than 10 to 20% of the heme intracellular levels[1], these data are consistent with the hypothesis that aromatase stimulation by dbT may involve *in part* the regulation of the intracellular heme supply and the subsequent insertion of heme into aromatase apocytochrome P-450 to activate the enzyme. Implicit in this unique hypothesis are the assumptions that intracellular heme is limiting and that aromatase apocytochrome P-450 is present -- assumptions supported by the rapid and significant aromatase stimulation by optimal concentrations of hemin in culture and *in vitro*. Since dbT stimulates aromatase to a

[1] Direct estimates of the total cytochrome P-450 in homogenized, subcellular-fractionated JAr cells were 3 and 13 pmol/mg protein in the low-speed pellet from control and dbT-grown cells, respectively, with undetectable levels (<2 pmol/mg protein) in the cell homogenate and microsomal fractions (8). Aromatase cytochrome P-450 constitutes only a fraction of the total cytochrome P-450. By extrapolating cytochrome P-450 levels from human placental microsomes to JAr cells based on the relative aromatase specific activity, we estimate aromatase cytochrome P-450 levels in JAr cells at about 0.1 and 1 pmol/mg protein in control and dbT-grown cells, respectively.

much greater extent than hemin, additional factors must be involved in the mechanism of dbT action.

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