Polyamines Control Human Chorionic Gonadotropin Production in the JEG-3 Choriocarcinoma Cell*

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The effect of inhibition of ornithine decarboxylase with difluoromethylornithine (DFMO) and the resultant lowering of polyamine levels upon human chorionic gonadotropin (hCG) production in JEG-3 choriocarcinoma cells was investigated. DFMO (10 mM) totally inhibited ornithine decarboxylase activity. In DFMO-treated cells, cellular spermidine concentrations fell to nondetectable levels (less than 1% of control values) within 24 h and spermine concentrations were reduced to 41.9% of controls over 6 days. DFMO caused a 70–80% inhibition of hCG production. Levels of mRNA for both the α and β subunits of hCG were also inhibited relative to mRNA for tubulin. Exogenous putrescine normalized hCG production in a dose-dependent manner. Other diamines, including cadaverine, 1,3-diaminopropane, 1,6-diaminohexane, and 1,7-diaminoheptane, were ineffective in reestablishing hCG production in DFMO-treated cells. Dibutyryl cAMP (1 mM) stimulated hCG production and increased levels of mRNA for the α and β subunit 5–40-fold in both DFMO-treated and control cells. Polyamines appear to have a fundamental role in hCG production in JEG-3 choriocarcinoma cells. However, dibutyryl cAMP can partially overcome or circumvent the requirement for polyamines in hCG biosynthesis.

Human chorionic gonadotropin (hCG), the major placental polypeptide hormone of early pregnancy is composed of an α subunit which is common with luteinizing and follicle-stimulating hormones and TSH and a unique β subunit (1–3). hCG production and release in placenta and choriocarcinoma is stimulated by a number of activators including cAMP analogs, phorbol 12-myristate 13-acetate, epidermal growth factor, and retinol (4–7). hCG stimulation by cAMP analogs has been extensively investigated; Weintraub and co-workers (8) and Nilson and co-workers (9) have demonstrated that cAMP, and phorbol esters have been shown to enhance ornithine decarboxylase expression in specific target tissues and cell lines (15–17). Because of the similarity between activators of ornithine decarboxylase activity and hCG biosynthesis, the similar changes in levels of polyamines and hCG with gestation, and the known rapid turnover of ornithine decarboxylase (11–13), we postulated that hCG production may be activated by polyamines.

DFMO, an enzyme-activated, irreversible inhibitor of ornithine decarboxylase was used to investigate the effect of polyamines upon hCG production in JEG-3 choriocarcinoma cells. Blockade of ornithine decarboxylase markedly inhibited hCG production. Bt2cAMP, however, was able to partially stimulate hCG production in the presence of ornithine decarboxylase blockade with DFMO.

EXPERIMENTAL PROCEDURES

Materials

DFMO was a generous gift of Merrill Dow Corp., Cincinnati, OH. L-[1,14C]Ornithine (61 mCi/mmol) was obtained from Amersham Corp. Bt2cAMP, sodium butyrate, dimethyl sulfoxide, putrescine (1,4-diaminobutane), spermidine, spermine, 1,3-diaminopropane, cadaverine (1,5-diaminopentane), 1,6-diaminohexane, and 1,7-diaminoheptane were obtained from Sigma. Fetal calf serum and other tissue culture materials were obtained from Hazeltown Research Products, Denver, PA.

Cell Culture

JEG-3 choriocarcinoma cells, originally cultured by Kohler and Birdson (18), were obtained from the American Type Tissue Collection in passage 126. Cells were grown in minimal essential medium with Earle's salts supplemented with 1 mM sodium pyruvate, nonessential amino acids, 10 mM Hepes (pH 7.2), 10% fetal bovine serum, and antibiotics (medium A) on Falcon (6-well) dishes until 80–90% confluent. At that time, experiments were initiated by setting up four groups: 1) medium A; 2) medium A plus 5.0 × 10−7 M putrescine; 3) medium A plus 10 mM DFMO; and 4) medium A plus 10 mM DFMO.
and 5.0 × 10^{-6} \text{ M} \text{ putrescine}. \text{ Experiments were generally run for 6 days. Media was changed daily. Triplicate samples of media and cells were saved for measurement of media hCG, and cellular hCG, polyamines, protein, and DNA, respectively. In indicated experiments BtzcAMP (1 mM) was added during the 5th and 6th culture day.}

**High-performance Liquid Chromatography Assay of Polyamines**

**Extraction and Dansylation—**After removal of the media, cells were washed twice with phosphate-buffered saline (pH 7.2) and then frozen in 1 ml of the phosphate-buffered saline buffer/well at −70 °C. A three-step extraction of the cell homogenate was added to 1.5 ml of 3.3% HClO4 plus 50 µl of the internal standard 1,7-diaminoheptane (52 µM). The sample was vortexed, placed on ice for 10 min, and then centrifuged at 20,000 × g for 20 min at 5 °C. A portion (1.4 ml) of the cell extract was added to 0.28 ml of 20% K2CO3/1.4 H2O (v/w) and placed on ice for 10 min. The supernatant (1.2 ml) was added to an additional 0.2 ml of K2CO3, mixed, and combined with 2.7 ml of acetone containing 2 mg/ml 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride). Samples were then sonicated for 10 min in an ultrasonic water bath, transferred to a 60 °C water bath, and incubated for 1 h.

The samples were diluted with 1 ml of water. Dansylated polyamines were then partially purified as described by Minchin and Hanau (19) employing Sep-Pak C18 cartridges (Waters Associates, Milford, MA) with the exception that dansylated polyamines were eluted from the C18 cartridges with 6 ml instead of 5 ml of 100% methanol.

**Separation of Polyamines—**Cells and dansylated polyamines in methanol (50 µl) were fractionated on a Beckman high-performance liquid chromatography system equipped with two model 112 pumps, a model 157 fluorescence detector (excitation and emission filters, 305–358 and 455–550, respectively), a model C-R1 recorder/integrator and model 420 system controller. The procedure employed reverse-phase chromatography utilizing a Partisil-10 ODS2 column (4.6 × 250 mm). At time 0 the column was equilibrated with 48% acetonitrile in water. One minute after injection a linear gradient to 90% acetonitrile was carried out over 20 min. The solvent was held at 90% acetonitrile for an additional 10 min and then recycled to 48% acetonitrile. The flow rate was 1 ml/min. An internal standard method based on one-point calibration (Beckman Manual No. 221-051A) employing authentic polyamines carried through the entire dansylation procedure was utilized to quantitate polyamines. Calibration curves were determined to be linear for putrescine between 2 and 150 nmol/50 µl and for spermidine and spermine between 0.5 and 150 nmol/50 µl injected.

**Ornithine Decarboxylase Assay**

At specified times cells were washed with 2.0 ml/well ice-cold phosphate-buffered saline followed by the addition of 1.5 ml of ornithine decarboxylase buffer (50 mM Tris-HCl, 0.4 mM EDTA, 100 mM pyridoxal phosphate, and 5 mM dithiothreitol at pH 7.4). The cells were removed with a rubber policeman and stored at −70 °C.

**Determination of mRNA Levels for the α and β Subunits of hCG**

Total cellular RNA was isolated by extraction with guanidine isothiocyanate and centrifugation through a cesium chloride step gradient (21). Concentration of RNA was determined by measuring the absorbance at 260 nm. Three micrograms of total cellular RNA from each plate were blotted directly on nitrocellulose paper (Schleicher & Schuell, Inc.) using a HybriSlot manifold (Bethesda Research Laboratories). Blots were baked, prehybridized, and hybridized to 32P-labeled single-stranded DNA probes complementary to α-hCG subunit, β-hCG subunit, and α-tubulin mRNAs (21). Filters were hybridized overnight, washed, and subjected to autoradiography for 2-8 h. Hybridization signals were quantitated by analyzing the film with a Zexneh soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA).

**RESULTS**

**Effect of DFMO on Ornithine Decarboxylase and Polyamines—**Ornithine decarboxylase activity was easily detectable in JEG-3 cells. Basal ornithine decarboxylase activity increased 3.5-fold with BtzcAMP and was suppressed with exogenous putrescine (5.0 × 10^{-6} \text{ M}) to 15-20% of basal levels (Table I). DFMO (10 mM), a highly specific enzyme-activated irreversible inhibitor of ornithine decarboxylase (for review see Ref. 24), effectively blocked ornithine decarboxylase in JEG-3 cells. BtzcAMP, an agent which stimulates increases in ornithine decarboxylase activity in JEG-3 (Table I) and other cell lines (16, 25), was unable to modulate ornithine decarboxylase in the presence of DFMO. An unexpected finding was the ability of BtzcAMP to stimulate ornithine decarboxylase activity 5-6-fold in the presence of usually inhibitory concentrations of putrescine. Putrescine is a potent inhibitor of ornithine decarboxylase and suppresses ornithine decarboxylase stimulation by hormones in most systems (11).

**Ornithine decarboxylase activity in JEG-3 cells**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>DFMO</th>
<th>Putrescine</th>
<th>DFMO + putrescine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.03 ± 0.033</td>
<td>ND</td>
<td>0.42 ± 0.03</td>
<td>ND</td>
</tr>
<tr>
<td>DFMO</td>
<td>ND</td>
<td>7.16 ± 0.064</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Putrescine</td>
<td>ND</td>
<td>1.28 ± 0.23</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Different from control in same treatment group, p < 0.001.

ND, nondetectable.

hCG β was measured using a commercial (Amersham Corp.) radioimmunoassay kit. Protein was measured using the method of Bradford (22). DNA was measured using the method of Burton (23).

**Other Assays**

*hCG μg was measured using a commercial (Amersham Corp.) radioimmunoassay kit. Protein was measured using the method of Bradford (22). DNA was measured using the method of Burton (23).**

**Ornithine decarboxylase activity in JEG-3 cells**

**Ornithine decarboxylase activity (nmol of CO2/h/mg of protein) was measured in control cells and cells treated with putrescine (5.0 × 10^{-6} \text{ M}), DFMO (10 mM), or both for 24 h. For each treatment group ornithine decarboxylase activity was also determined with and without BtzcAMP (1 mM). (n = 3, mean ± S.D.)**

**Table I**

**Ornithine decarboxylase activity in JEG-3 cells**

**Ornithine decarboxylase activity (nmol of CO2/h/mg of protein) was measured in control cells and cells treated with putrescine (5.0 × 10^{-6} \text{ M}), DFMO (10 mM), or both for 24 h. For each treatment group ornithine decarboxylase activity was also determined with and without BtzcAMP (1 mM). (n = 3, mean ± S.D.)**
Polyamines and hCG Production

Conlrol DFMO Putrescine D+P

**FIG. 1. Effect of DFMO on polyamine levels.** Intracellular spermidine (A) and spermine levels (B) (nmol/µg of DNA) are shown for the final day of a 6-day experiment in control cells and in cells treated with DFMO (10 mM), putrescine (5.0 × 10^{-5} M), or both (D + P). The mean ± S.D. of three separate experiments is shown.

controls after 6 days in culture. Four to six days in culture with DFMO was required to achieve the maximal hCG inhibition. This time period also allowed intracellular levels of polyamines to be depleted and growth of both control and DFMO-treated cells to plateau. With 6 days of DFMO treatment, hCG production was inhibited 80% relative to control levels. Exogenous putrescine (5.0 × 10^{-5} M) completely reversed the effect of DFMO upon hCG production (Fig. 2).

The dose dependence of hCG production upon putrescine in DFMO-treated cells is shown in Fig. 3. Putrescine at 10^{-6} M was required to attain control levels of hCG production in DFMO-treated cells. The specificity for putrescine was determined using structural analogs. Structural analogs of putrescine (all at 5.0 × 10^{-5} M) were added to DFMO-treated cells over a 5-day period (Fig. 4). None of the other tested diamines were able to substantially reverse the DFMO inhibition of hCG. A small increase in hCG production above that seen in DFMO-treated cells was produced with cadaverine. Suppression of hCG production below the level in DFMO-treated cells was seen with 1,6-diaminohexane and 1,7-diaminoheptane.

**Effect of DFMO upon mRNA Levels of the α and β Subunits**—DFMO reduced total cellular RNA to 26% of control levels after 6 days in culture. When equal amounts of RNA from cells treated with DFMO and controls were examined, however, the relative amounts of α and β subunit mRNA were 11- and 4.6-fold less, respectively, than controls. Tubulin mRNA levels in DFMO-treated cells were much less depressed relative to controls, suggesting that DFMO specifically inhibits hCG (Fig. 5).

**Requirement for Polyamines in the Stimulation of hCG by Bt2cAMP—**Bt2cAMP (1 mM) increased spermidine levels. Spermine levels were not statistically altered (using analysis of variance) by Bt2cAMP (Fig. 6). Bt2cAMP (1 mM) increased hCG production 10-fold above that in control cultures (Fig. 7). In DFMO-treated cultures, the addition of Bt2cAMP also stimulated hCG production 5-6-fold over basal hCG production in DFMO-treated cells. hCG levels comparable to those produced in cells treated with Bt2cAMP alone were not reached in the cultures containing DFMO, however. In the

**FIG. 2. Effect of DFMO on hCG production.** hCG (ng/mg of protein/24 h) is shown for the final day of a 6-day experiment in control cells and in cells treated with DFMO (10 mM), putrescine (5 × 10^{-5} M), or both (D + P). The mean ± S.D. of three separate experiments is shown.

**FIG. 3. Putrescine dose response.** hCG (ng/mg of protein/24 h) is shown for the final day of a 3-day experiment in control cells, in cells treated with DFMO (10 mM), or in cells treated with DFMO (10 mM) plus putrescine of increasing concentrations (10^{-8}-10^{-6} M). All cultures, except for controls, were incubated with DFMO (10 mM) for 2 days prior to initiation of the 3-day experiment. The mean ± S.D. of three separate experiments is shown.

**FIG. 4. Effect of putrescine analogs upon DFMO-treated cells.** hCG (ng/mg of protein/24 h) is shown for the final day of a 6-day experiment in control cells, in cells treated with DFMO (10 mM), in cells treated with putrescine (5.0 × 10^{-5} M), or in cells treated with DFMO plus one of the putrescine analogs (cadaverine, 1,3-diaminopropane (1,3-Dp), 1,6-diaminohexane (1,6-Dh), or 1,7-diaminoheptane (1,7-Dh); all at 5.0 × 10^{-5} M). The mean ± S.D. of three separate experiments is shown.
TABLE II  
Effects of DFMO on hCG mRNA

<table>
<thead>
<tr>
<th>Treatments</th>
<th>α-hCG</th>
<th>β-hCG</th>
<th>α-Tubulin</th>
<th>μg/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.11 ± 0.03</td>
<td>0.55 ± 0.05</td>
<td>0.26 ± 0.03</td>
<td>37.3 ± 2.3</td>
</tr>
<tr>
<td>DFMO</td>
<td>0.01 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>9.6 ± 1.0</td>
</tr>
<tr>
<td>cAMP</td>
<td>4.25 ± 0.34</td>
<td>4.55 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>31.1 ± 0.6</td>
</tr>
<tr>
<td>DFMO + cAMP</td>
<td>0.38 ± 0.16</td>
<td>0.65 ± 0.28</td>
<td>0.13 ± 0.02</td>
<td>10.0 ± 1.1</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of DFMO upon hCG α and β mRNA. Autoradiograph of slot blot hybridization of 32P-labeled cDNA probes for α-hCG, β-hCG, and α-tubulin to total cellular RNA in control cells and in cells treated with DFMO (10 mM) for 6 days. On days 5 and 6 of the 6-day incubation, indicated cultures were additionally treated with Bt2cAMP (1 mM). Total RNA (μg/dish) for each treatment group is also given. (n = 3, mean ± S.D.)

Fig. 6. Effect of Bt2cAMP upon polyamine levels. Intracellular spermidine and spermine levels (nmol/μg of DNA) are shown for the final day of a 6-day experiment in control cells and in cells treated with DFMO (10 mM), putrescine (5.0 × 10⁻⁵ M), or both for 6 days. During days 5 and 6 of the 6-day incubation, indicated cultures were treated with Bt2cAMP (1 mM). The mean ± S.D. of three separate experiments is shown.

The presence of both DFMO and putrescine, Bt2cAMP stimulated hCG production above that seen with Bt2cAMP alone (Fig. 7). Butyrate (1 mM) inhibited hCG production in all cultures (data not shown).

Changes in mRNA for both the α and β subunit of hCG paralleled the protein changes. Bt2cAMP increased α and β subunit mRNA 41.5- and 8.27-fold, respectively. In the presence of both DFMO, Bt2cAMP increased the relative proportion of α and β subunit mRNA approximately similarly above that seen with DFMO alone (Fig. 5, Table II). The α subunit mRNA was stimulated to a greater extent than the β subunit mRNA by cAMP both in the presence and absence of DFMO. As with hCG protein levels, however, Bt2cAMP did not stimulate hCG mRNA in DFMO-treated cultures to levels comparable to those produced by Bt2cAMP alone. Full stimulation of hCG production by this second messenger measured either at the protein or mRNA level was not seen when polyamines were depleted with DFMO.

DISCUSSION

The central finding of this study is that polyamines are required for basal production of hCG in JEG-3 choriocarcinoma cells and are also required for maximal stimulation of hCG by cAMP.

DFMO affected both growth and hCG production. The effect of DFMO blockade of ornithine decarboxylase upon hCG, however, was much greater than its effect upon cell growth. hCG output per milligram of protein or microgram of DNA was 5-fold lower in DFMO-treated cells than in controls. Also, hCG output was significantly less when DFMO cells were compared with control cells at equal density. JEG-3 cells in DFMO continue to grow to confluency and survive replating at least two times while producing minimal hCG. Thus, production of proteins and DNA replication required for growth and division continues for some time with DFMO.

Finally, mRNA levels for both subunits of hCG are specifically inhibited by DFMO relative to mRNA for tubulin. Therefore, although DFMO has an effect upon hCG through its general effect upon growth, DFMO inhibition of hCG production is more pronounced relative to other cell proteins. Although this is the first report of specific inhibition of hCG production by polyamine depletion, some specificity of the effect of polyamine depletion upon mRNA production has been previously reported by Amri et al. (26). In the preadipocyte cell line, Amri et al. reported that polyamine depletion reduced levels of mRNA for glyceraldehyde-3-phosphate dehydrogenase but not for lipoprotein lipase. Polyamine biosynthesis has also been shown to be required for the synthesis of specific proteins in other biological systems. In a series of reports it was shown that α-lactalbumin and casein synthesis are markedly reduced in mouse mammary gland when polyamine biosynthesis is inhibited (for review see Ref. 27).
Our data confirm the stimulation of hCG production by Bt2cAMP. In the presence of DFMO Bt2cAMP was also able to stimulate hCG and the mRNAs for the α and β subunits 5-10-fold above the level observed with DFMO alone. In both control and DFMO-treated cells, Bt2cAMP stimulated the α subunit mRNA to a greater extent than the β subunit mRNA. Burnside et al. (8) and Milsted et al. (9) have both previously described differential regulation of hCG α and β subunits by cAMP with relative-fold stimulation of subunit mRNA dependent upon duration of stimulation and culture conditions. The effects of Bt2cAMP must be viewed in the context that final levels of hCG and subunit mRNA attained in DFMO-treated cells were well below that seen in cultures treated with Bt2cAMP alone. The ability of Bt2cAMP to overcome the effect of DFMO blockade upon hCG production is therefore only partial, and polyamines must be considered necessary for attainment of the full effect of cAMP. Furthermore, these data demonstrate that Bt2cAMP is effective in stimulating both the α and β hCG mRNA in DFMO-treated cells (Fig. 5, Table II). It is therefore unlikely that activation of polyamine production by increasing ornithine decarboxylase activity is an intermediary step which might account for differences in the transcription kinetics of the α and β hCG subunit genes when stimulated by Bt2cAMP (8, 9).

DFMO blockade of ornithine decarboxylase activity in JEG-3 cells results in rapid depletion of spermidine and slower partial depletion of spermine. Cell growth is inhibited significantly by DFMO, but hCG and hCG mRNA are greatly inhibited even after normalization for differences in growth. Bt2cAMP can partially overcome the effect of DFMO blockade of ornithine decarboxylase upon production of hCG and its subunit mRNA. The data are consistent with a major role of polyamines in hCG production and its stimulation above basal levels by cAMP. The mechanism by which polyamines exert effects on hCG is unknown, but further study of this system may provide insight into the action of polyamines and their potential interplay with major hormone systems in the trophoblast.

REFERENCES