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Inhibition of hormone-stimulated steroidogenesis in cultured Leydig tumor cells by a cholesterol-linked phosphorothioate oligodeoxynucleotide antisense to diazepam-binding inhibitor

(trophic hormones/steroids)

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ABSTRACT **The polypeptide diazepam-binding inhibitor (DBI) has been previously shown to stimulate testicular Leydig, adrenocortical, and glial-cell mitochondrial steroidogenesis *in vitro*. To assess the *in situ* role of DBI in trophic hormone-stimulated steroidogenesis, we suppressed DBI levels in the hormone-responsive MA-10 Leydig tumor cells, using a cholesterol-linked phosphorothioate oligodeoxynucleotide (Chol–odN) antisense to DBI. Treating MA-10 cells with Chol–odN antisense to DBI resulted in a dose-dependent reduction of DBI levels (ED_{50} = 1 μM). In contrast, Chol–odN sense to DBI did not affect its expression. Saturating amounts of human choriogonadotropin (hCG) increased MA-10 progesterone production by 150-fold. Addition of increased concentrations of Chol–odNs sense to DBI or of a nonrelated sequence did not reduce the MA-10 response to hCG. However, in the presence of Chol–odN antisense to DBI that could reduce DBI levels, MA-10 cells lost their ability to respond to hCG (ED_{50} = 1 μM). In these studies the hCG-stimulated cAMP levels and cytochrome P450 side-chain cleavage activity, as measured by metabolism of 22(*R*)-hydroxycholesterol, were not affected by the Chol–odNs used. These observations provide unequivocal evidence that DBI plays a vital role in the acute stimulation of steroidogenesis by trophic hormones.**

The primary point of control in the acute stimulation of steroidogenesis by trophic hormones involves the first step in this biosynthetic pathway, where cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage cytochrome P450 ($P450_{scc}$) and auxiliary electron-transferring proteins localized on the inner mitochondrial membranes (1–4). More detailed studies have shown that the reaction catalyzed by $P450_{scc}$ is not rate-determining in the synthesis of steroid hormones, but rather it is the transport of the precursor, cholesterol, from intracellular stores to the inner mitochondrial membrane where steroid production begins (1–4). This hormone-dependent transport mechanism was shown to be mediated by cAMP and to be sensitive to the protein synthesis inhibitor cycloheximide (1–4). Thus, the concept of a labile intracellular—presumably a cytoplasmic protein—mediator of hormone action on steroidogenic tissues arose (1–4).

In search of such cytoplasmic steroidogenesis-stimulating factor(s), a number of proteins have been identified as potential candidates (5–8), but no direct evidence for their *in situ* role has yet been provided. More recently a protein has been isolated from bovine adrenals (9, 10) that stimulates transport of cholesterol into mitochondria, transport from the outer to the inner membrane, and promotes loading of $P450_{scc}$ with substrate (cholesterol) (9, 10). This protein was shown to be identical to the polypeptide diazepam-binding inhibitor (DBI) (11), except for the loss of two amino acids (Gly-Ile) from the carboxyl terminus (12). DBI was originally purified from brain by monitoring its ability to displace diazepam from the allosteric modulatory sites for γ-aminobutyric acid action on γ-aminobutyric $acid_A$ receptors (11, 13). DBI was found to be present in a variety of tissues (14), to be highly expressed in adrenocortical and testicular Leydig (14–16) steroid-producing cells, and to have a variety of specialized functions in different tissues (10, 11, 13, 15, 16–18). We and others (9, 10, 15, 16, 19, 20) then showed that bovine adrenal $Des\text{-}Gly^{85}\text{-}Ile^{86}$-DBI, rat and bovine brain native DBI, as well as rat testis native DBI stimulate intramitochondrial cholesterol transport and increase pregnenolone formation by isolated adrenocortical, Leydig, or glial-cell mitochondria. In conjunction with these findings, a drug-binding site, the mitochondrial peripheral-type benzodiazepine/DBI receptor (PBR), was shown to regulate cholesterol delivery to the inner membrane (12, 19, 21–24). Furthermore, the action of DBI on mitochondrial steroidogenesis was shown to be mediated by PBR in all tissues examined thus far (12, 15, 19, 20).

Although the *in vitro* role of DBI on mitochondrial steroidogenesis seems well established, there is no information about the *in situ* role of DBI in hormone-stimulated steroidogenesis. Thus, the studies presented here were designed to determine whether DBI is implicated in the acute trophic hormone-stimulated steroid biosynthesis.

MATERIALS AND METHODS

Materials. Purified human chorionic gonadotropin (hCG) (batch CR-125 of biological potency 11,900 international units/mg) was from the National Institutes of Health. The synthesis of phosphorothioate oligonucleotides (odNs) was based on the published murine DBI sequence (25). Cholesterol-linked odNs (Chol–odNs) antisense (5′-cholesterol-TTCAGCCTGAGACATACTGGTGAG-3′) or sense (5′-cholesterol-AAGTCGGACTCTGTATGACCACTC-3′) to DBI or of a nonrelated sequence (5′-cholesterol-CGCGC-CCCGCCGCCGCCATG-3′) were produced by using β-cyanoethyl phosphoramidate chemistry with a sulfurizing reagent (26) in place of the normal oxidizer to produce the phosphorothioate backbone (27). A cholesterol phosphoramidite was used to add a single cholesterol functionality to the 5′ end (27). odNs were purified by reverse-phase chromatography.

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Abbreviations: DBI, diazepam-binding inhibitor; hCG, human chorionic gonadotropin; PBR, peripheral-type benzodiazepine/DBI receptor; $P450_{scc}$, C_{27} cholesterol side-chain cleavage cytochrome P450; odN, phosphorothioate oligodeoxynucleotide; Chol–odN, cholesterol-linked odN.

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Cell Culture. MA-10 mouse Leydig tumor cells were from Mario Ascoli (University of Iowa) and were plated at low density for 24 hr in modified Waymouth's MB 752/1 medium/20 mM Hepes/$NaHCO_3$ at 1.2 g/liter/15% horse serum, pH 7.4 (21, 28). After 24 hr the medium was changed, and fresh medium containing the indicated amounts of Chol–odNs or cholesterol was added for 48 hr. At the end of incubation the cells were used to determine DBI levels or they were washed with serum-free medium and stimulated for 2 hr with saturating amounts of hCG (1 nM) or 22(*R*)-hydroxycholesterol (30 μM) in serum-free medium. At the end of the 2-hr incubation period the cell medium was saved for progesterone determination, and the cells were either dissolved in 0.1 M NaOH for protein determination or processed for measurement of both protein and intracellular cAMP levels.

Determination of DBI Levels. DBI levels were determined by immunoblot analysis of MA-10 cell sodium acetate extracts enriched in DBI by Sep-Pak C_{18} chromatography, as we described (29). Proteins were separated by SDS/PAGE with the method of Fling and Gregerson (30), transferred to nitrocellulose, and then immunoblotted by using a specific anti-DBI antiserum (13, 29); DBI levels were then quantified by densitometry.

Radioimmunoassays. Progesterone production by MA-10 cells was measured by means of RIA (21). Intracellular cAMP levels were determined by RIA in ethanol-trichloroacetic acid cell extracts, as described (31).

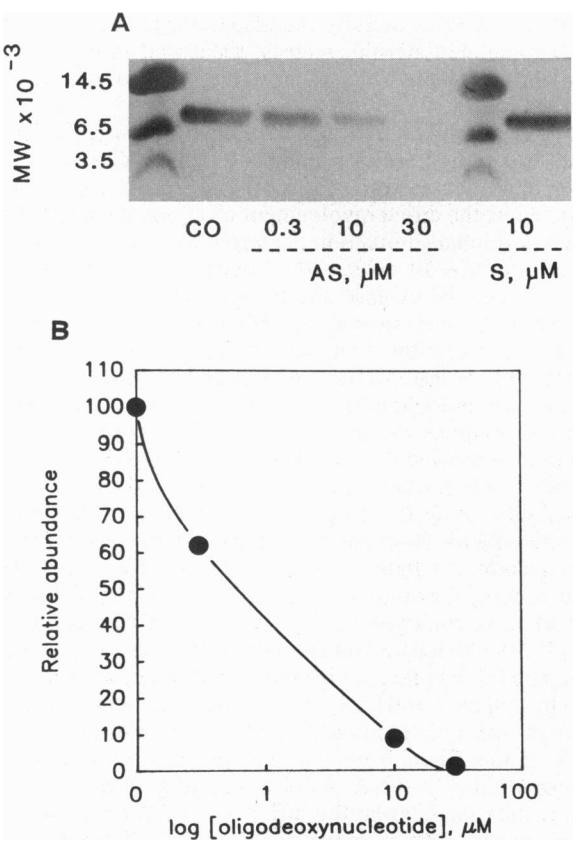

FIG. 1. Chol–odNs antisense but not sense to DBI inhibit DBI expression in MA-10 Leydig cells. MA-10 cells were plated in 6-well plates, as described. After 24 hr the medium was changed, and fresh medium containing the indicated amounts of Chol–odNs was added for 48 hr. DBI was extracted and partially purified; its levels were determined by immunoblot analysis. CO, control; AS, antisense; S, sense; MW, M_r. (*A*) Immunoblot. (*B*) DBI levels are quantitated on the ordinate.

Protein Measurement. Protein was measured by the method of Bradford (32), using bovine serum albumin as a standard.

Statistics. Statistical analysis was done by one-way ANOVA.

RESULTS AND DISCUSSION

To determine the *in situ* role of DBI in hormone-induced steroid biosynthesis, the hormone-responsive MA-10 mouse tumor Leydig cell line (28), previously shown to be an excellent model to study the role of PBR and DBI in steroidogenesis (15, 21–23), was used. To overcome the usually encountered oligodeoxynucleotide-uptake problems (33) and knowing that steroidogenic cells, including the MA-10 cells, can use exogenous cholesterol via the lipoprotein endocytotic pathway (34, 35) we constructed Chol–odNs (27) in which cholesterol could act as a carrier to facilitate their uptake.

Chol–odNs complementary to either the sense or the antisense strand of the 24 nt encoding mouse DBI (25), 9 bases immediately 5′ to the initiator codon ATG and 12 downstream the ATG codon were constructed (26, 27). Treating MA-10 cells with Chol–odN antisense to DBI resulted in a dose-dependent reduction (>90%) of DBI levels (Fig. 1*A*). Quantitation of the DBI levels determined by immunoblot analysis indicated that 50% reduction of DBI levels was obtained with ≈1 μM Chol–odN antisense to DBI (Fig. 1*B*). In contrast, Chol–odN sense to DBI did not affect its expression.

We then examined the effect of hormone stimulation on MA-10 steroidogenesis. Saturating amounts of hCG (1 nM) increased progesterone production by 150-fold. Addition of increased concentrations of Chol–odNs sense to DBI or of a nonrelated sequence did not reduce the MA-10 cell response to hCG (Fig. 2). In contrast, a 2-fold increase in the amount of steroids produced was observed, probably due to the cholesterol linked to the odN, liberated in the cells, and used

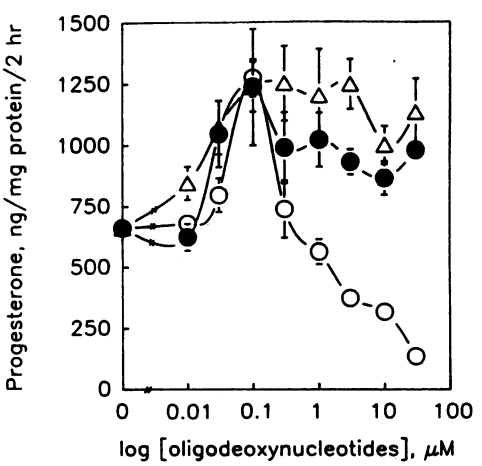

FIG. 2. Chol–odNs antisense but not sense to DBI or of a nonrelated sequence inhibit trophic hormone-stimulated steroid synthesis by MA-10 Leydig cells. MA-10 cells were plated in 24-well plates as described. After 24 hr the medium was changed, and fresh medium containing the indicated amounts of Chol–odNs antisense to DBI (○), sense to DBI (●), or of a nonrelated sequence (△) was added for 48 hr. The cells were then washed with serum-free medium and stimulated for 2 hr with saturating amounts of hCG (1 nM). At the end of the incubation period, the cell medium was saved, and progesterone was measured by RIA. Under basal conditions MA-10 cells secrete 4.37 ± 0.45 ng of progesterone per mg of protein per 2-hr incubation period. Results are means ± SDs. (n = 6). Similar results were obtained in two other independent experiments on cells of different passages.

Table 1. Effect of cholesterol on hCG-stimulated steroidogenesis

Cholesterol treatment	hCG-stimulated steroidogenesis, ng of progesterone per mg of protein
None	1256 ± 92
0.04 μM	1516 ± 156
0.13 μM	1517 ± 159
0.43 μM	1915 ± 278
1.30 μM	1982 ± 229
4.30 μM	2041 ± 70
13.0 μM	1733 ± 99
43.0 μM	1888 ± 144
130.0 μM	1836 ± 256

Cells were plated in 24-well plates and treated with the indicated amounts of cholesterol, as described. At the end of incubation, cells were washed and stimulated for 2 hr with hCG (1 nM). Progesterone secreted into the medium was then measured by RIA. Means ± SDs of triplicate determinations are presented; similar results were obtained in two other independent experiments on cells of different passages.

as substrate for steroid synthesis. This hypothesis is supported by data presented in Table 1, where increased concentrations of cholesterol, similar to those present in the Chol–odNs used, potentiated the stimulatory effect of hCG. A similar increase of the amount of steroids produced by MA-10 cells treated with 30–100 nM Chol–odN antisense to DBI was also seen. However, in the presence of higher concentrations of Chol–odN antisense to DBI, able to reduce DBI levels in the cells (Fig. 1), MA-10 cells lost their ability to respond to hCG; 50% inhibition was obtained by using 1 μM antisense odNs (Fig. 2). This effect of Chol–odN antisense to DBI on hCG-stimulated steroidogenesis was found to be extremely significant ($P < 0.0001$) by ANOVA.

The observation that the potency of Chol–odN antisense to DBI to reduce DBI levels and to inhibit hCG-induced steroidogenesis is comparable (1 μM) provides strong evidence that the effect of Chol–odN antisense to DBI on hormone-stimulated steroidogenesis is consequent to its effect on DBI expression. It should be noted that the potency of Chol–odN antisense to DBI to inhibit DBI protein expression is 10–30 times higher than that of other types of odNs (ref. 33; IC_{50} = 10–30 μM) used to inhibit protein expression. It is evident that cholesterol greatly facilitated the odN uptake via the

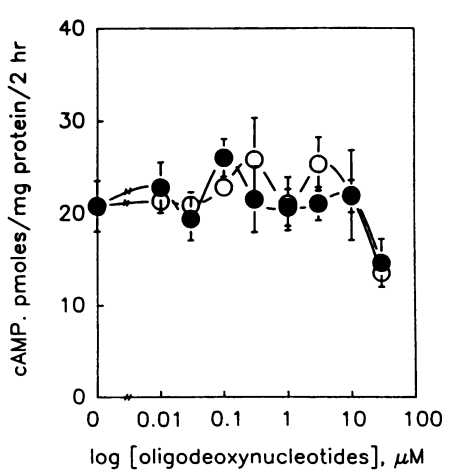

FIG. 3. Effect of Chol–odNs, antisense (○) and sense (●) to DBI, on hCG-stimulated cAMP synthesis. MA-10 cells were plated and treated as described for Fig. 2. Intracellular cAMP levels were determined by RIA. Basal values were 7.80 ± 1.27 pmol/mg of protein per 2 hr. Results are means ± SDs ($n = 6$). Similar results were obtained in two other independent experiments on cells of different passages.

Table 2. Effect of Chol–odNs on hCG- and 22(*R*)-hydroxycholesterol-stimulated steroidogenesis

Chol–odN treatment	Progesterone production, ng/mg of protein	
	hCG*	22(*R*)-Hydroxycholesterol*
None	917.3 ± 139.2	1441.6 ± 198.5
Antisense	83.6 ± 16.5	1569.4 ± 330.0
Sense	1644.7 ± 42.2	1896.6 ± 365.6
Nonrelated	1272.3 ± 138.5	1468.7 ± 435.1

Cells were plated in 24-well plates and treated with the indicated Chol–odNs (10 μM), as described. At the end of incubation, cells were washed and stimulated for 2 hr with hCG (1 nM) or 22(*R*)-hydroxycholesterol (30 μM). Progesterone secreted into the medium was then measured by RIA. Means ± SDs of triplicate determinations are presented; similar results were obtained in a separate experiment on cells of different passages.
*hCG- or 22(*R*)-hydroxycholesterol-stimulated steroidogenesis, respectively.

active lipoprotein-mediated endocytotic pathway. This approach may be useful to other cell systems for studying the role of specific proteins in cell functions.

An important control in these studies was the hCG-dependent intracellular cAMP accumulation that was not affected by the Chol–odNs used (Fig. 3). In our studies the cAMP values obtained closely resemble reported values for this cell model (36). Our second control was to determine the $P450_{scc}$ activity, measured by metabolism of the water-soluble cholesterol analogue 22(*R*)-hydroxycholesterol, which can cross cell membranes and directly access the $P450_{scc}$ in the inner mitochondrial membrane. As shown in Table 2 the $P450_{scc}$ activity was also found intact, whereas the hCG-stimulated steroidogenesis, measured in the same experiments by using cells of the same passage, was dramatically reduced.

These data unequivocally demonstrate that DBI plays a vital role in the hormone-induced cascade of events leading to increased cholesterol transport and steroid synthesis. We thus report the direct involvement of a nonenzyme protein in acute hormonal stimulation of steroidogenesis. The observations that MA-10 cells with reduced levels of DBI have normal adenylyl cyclase and $P450_{scc}$ activities, whereas the hormone-induced steroid synthesis has been suppressed, further support the concept of DBI involvement in the process of cholesterol transport from the intracellular stores to the inner mitochondrial membrane, as shown in studies in cell-free systems (9, 10, 15, 19, 20). However, in previous studies we showed that DBI has a half life of 3–6 hr and is not induced by hormones or cAMP (29), thus suggesting that the possibility of DBI being the long-sought cycloheximide-sensitive factor (1–4) can be excluded. Nevertheless, taking into account the findings that (*i*) DBI is preferentially localized around the mitochondria (37), (*ii*) it stimulates mitochondrial steroidogenesis (9, 10, 15, 19, 20) acting via PBR (12, 15, 19, 20) located on the outer mitochondrial membrane (38), and (*iii*) its presence is vital for induction of steroidogenesis by trophic hormones, we propose a model where the site of hormone action is placed at the interaction of DBI with PBR. In this model trophic hormones induce conformational changes either in PBR or its mitochondrial microenvironment, thus modulating the affinity of PBR for DBI, which then triggers intramitochondrial cholesterol transport and subsequent steroid formation.

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