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ETHANOL ENHANCEMENT OF LIGAND-STIMULATED cAMP PRODUCTION BY CULTURED HUMAN PLACENTAL TROPHOBLASTS

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Abstract—Chronic ethanol (EtOH) use during pregnancy can be associated with fetal injury including the fetal alcohol syndrome (FAS). A contributing factor in this fetal injury may be the effect of EtOH on the placenta. In this study, we have examined the effect of *in vitro* EtOH treatment on adenosine 3′:5′-cyclic monophosphate (cAMP) production by cultured trophoblasts, in response to various ligands. Epinephrine (10^{-6} M) rapidly stimulated cAMP with a peak between 2.5 and 5 min, which gradually returned to basal levels over 3–4 hr. EtOH treatment for >16 hr resulted in an up-regulation of epinephrine-stimulated cAMP production. Inhibition of phosphodiesterase with Rolipram® enhanced the effect of EtOH on cAMP production, suggesting that the effect of EtOH treatment was not due to phosphodiesterase inhibition. In cultured trophoblasts, EtOH treatment increased both epinephrine and 16,16′-dimethylprostaglandin E_2 (dm-PGE_2)-dependent cAMP production at varying ligand concentrations, suggesting an increased capacity to respond. When trophoblasts were treated with forskolin, a stimulator of adenylyl cyclase, cAMP production was enhanced in EtOH-treated cells. This suggests that EtOH treatment enhances adenylyl cyclase activity in these intact, cultured cells. Unlike trophoblasts from term human placenta, JAR choriocarcinoma cells did not respond to epinephrine, adenosine, or dm-PGE_2. The choriocarcinoma cells appeared to have lost the ability to respond to these ligands. Although the JAR cell adenylyl cyclase was stimulated by forskolin, EtOH treatment did not alter forskolin-stimulated cAMP production. In summary, EtOH-induced up-regulation of cAMP production appears to be cell specific, being present in normal human trophoblasts but not in undifferentiated choriocarcinoma cells.

Key words: ethanol; cAMP production; human placenta; trophoblast

Chronic EtOH† use during pregnancy is associated with fetotoxicity and can lead to the development of fetal alcohol syndrome (FAS) [1, 2]. Although the mechanism of EtOH toxicity is not known, both direct and indirect actions on the fetus may be involved. One indirect mechanism may be altered placental function. Many placental functions are critical for normal fetal growth and development. Previous studies in this and other laboratories suggest that EtOH may adversely affect placental nutrient transport [reviewed in Refs. 3 and 4] and cAMP-dependent hormone production [5]. EtOH may interfere with placental transport by altering the intracellular regulatory mechanisms that modulate trophoblast function. Although those trophoblast regulatory mechanisms that are EtOH-sensitive remain to be elucidated, cellular signal transduction pathways have been identified as EtOH-sensitive in other tissues [reviewed in Refs. 6 and 7]. Both *in vivo* and *in vitro* exposures to EtOH have been shown to affect signal pathways such as phospholipase C and adenylyl cyclase. "Acute" exposure to EtOH appears to either increase [8–11] or decrease [10, 12] ligand-mediated cAMP production. "Chronic" EtOH exposure also appears to either increase [13–15] or decrease [7, 15–19] ligand-stimulated cAMP production in both a tissue and ligand-specific manner. Recently, we have shown that continuous exposure of cultured human placental trophoblasts to EtOH for 24 or 72 hr enhances adenosine-stimulated cAMP production [5]. In this study, we describe the effect of continuous EtOH exposure on ligand-stimulated cAMP production in cultured human placental trophoblasts. The results suggest an increase in adenylyl cyclase activity.

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† Abbreviations: EtOH, ethanol; cAMP, adenosine 3′:5′-cyclic monophosphate; dm-PGE_2, 16,16′-dimethyl-prostaglandin E_2; VIP, vasoactive intestinal peptide; DMEM, Dulbecco's Modified Eagle's Medium; hCG, human chorionic gonadotropin; FBS, fetal bovine serum; and PCA, perchloric acid.

MATERIALS AND METHODS

Materials. DMEM, FBS, and calcium-magnesium (Ca^{2+}-Mg^{2+})-free Hanks' buffer were obtained from Gibco (Grand Island, NY) or Biofluids (Rockville, MD). Dispase was purchased from either Collaborative Research (Bedford, MA) or Boehringer–Mannheim (Indianapolis, IN). Rolipram® was provided by Dr. M. E. Sullivan of Berlex Laboratories (Cedar Knolls, NJ). Assay kits were obtained as follows: cAMP from either the Diagnostic

Products Corp. (Los Angeles, CA) or Amersham (Arlington Heights, IL); and hCG from the Ciba-Corning Diagnostics Corp. (Medfield, MA). Percoll, epinephrine, norepinephrine, dm-PGE_2, adenosine, glucagon, VIP and routine chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO).

Trophoblast purification. Normal term placentas were obtained within 15 min of either vaginal or Caesarean delivery and processed immediately for trophoblast isolation [20], as modified in this laboratory [5, 21, 22]. Purified trophoblasts concentrated between densities 1.064 and 1.051 on a discontinuous Percoll gradient. These cells were removed and washed with DMEM containing 25 mM HEPES and centrifuged at 200 *g* for 5 min.

Trophoblast culture. Purified cytotrophoblasts were diluted in DMEM containing 4 mM glutamine, 50 μg/mL gentamicin, and 20% heat-inactivated FBS. The cells were plated at a density of 1.5×10^5 cells/cm^2 into Falcon Primaria® culture wells, either 35-mm dishes (1 mL medium) or 24-well cluster plates (0.5 mL) (Becton Dickinson, Lincoln Park, NJ), and incubated in humidified 5% CO_2 at 37°. The medium was changed every 24 hr. At the time of plating, all cells were mononuclear and relatively uniform in size. Previous studies had shown that the initial purification methods yield approximately 95% pure trophoblasts [5, 21, 22]. Upon inoculation, cells attach and spread within 12 hr. As has been reported by Kliman *et al.* [20], phase contrast microscopy confirmed that the majority of trophoblasts from multinucleated syncytia within 12–24 hr of plating. This morphologic differentiation was concurrent with the appearance of hCG in the medium. Within the first 24-hr period, measurable amounts of hCG appeared in the culture medium. This was accompanied by uniform immunocytochemical detection of cytoplasmic hCG in all attached cells by 24 hr. At 24 hr, no cells stained for the non-trophoblast marker, vimentin, in either control or EtOH-treated cultures.

On the day of harvest, the medium was centrifuged to remove debris, and the supernatant was stored at −20° until assayed for hormones. The cells were washed twice with cold PBS. The washed cells were digested in 0.2 N NaOH containing 0.2% SDS for 30 min with agitation. The digest was stored at −20° until assayed for protein.

Continuous EtOH treatment. Continuous EtOH treatment [5] used a sealed chamber (Modular Incubator Chamber, Billups-Rothenberg, Del Mar, CA, or plastic dessicator, VWR Scientific, Piscataway, NJ) equilibrated with a 5% CO_2:95% air mixture. Briefly, an EtOH solution was placed in a tray at the bottom of the chamber. The concentration of EtOH in the reservoir tray was 800 mg/dL at the beginning of each 24-hr period. Incubation at 37° resulted in vaporization of EtOH, which gradually diffused into the culture medium. Control cells were also maintained under similar conditions except that H_2O was added to the reservoir tray. This method resulted in accumulation of EtOH in the culture medium reaching approximately 180 mg/dL (39 mM) in 8 hr and a plateau from 16 to 24 hr at approximately 280–300 mg/dL (61–65 mM), as determined by the alcohol dehydrogenase method (Behring Diagnostics, Somerville, NJ). In all experiments, the culture medium was replaced with fresh medium at 24 hr intervals. The EtOH solution in the reservoir was also changed at 24-hr intervals. This method resulted in cyclical exposure of the cells to EtOH. The peak concentration of 300 mg/dL (65 mM) is found in some chronic alcoholics [23] and even some alcoholic women at the time of delivery [24]. In experiments to determine the time course for the effect of EtOH exposure on ligand-stimulated cAMP production, EtOH was added directly to the culture medium at 300 mg/dL (65 mM) before placing the cells in the Modular Incubator.

Hormone-stimulated cAMP production. Trophoblasts were cultured in 24-well cluster plates for up to 3 days, with or without EtOH exposure. To determine hormone-induced cAMP production in these experiments, the culture medium was replaced with 400 μL of isotonic buffer (10 mM HEPES, 150 mM NaCl, 0.8 mM $MgSO_4$, 1.2 mM $CaCl_2$, 0.86 mM K_2HPO_4, 0.14 mM KH_2PO_4, pH 7.4) and incubated at 37° for 10 min. The medium was changed to 200 μL fresh buffer containing 10 μM Rolipram. After 5 min of additional incubation, test hormone was added to the desired concentration. The reaction was usually terminated at 10 min by adding 20 μL of 5 M PCA, and the sample was placed on ice and extracted for 30 min with agitation (90 rpm; Orbit Shaker, Lab-line Instruments, Inc., Melrose Park, IL). The extracts were transferred into microfuge tubes, each well was washed with 0.1 mL of 0.5 M PCA, and extracts were combined. The extract was neutralized with 25% KOH/30% K_2HCO_3. Following removal of $KClO_4$, 150 μL of the supernatant was assayed for cAMP content using a competition binding assay or radioimmunoassay using kits from the Diagnostic Products Corp. or Amersham, respectively. Any changes in these procedures are detailed in the figure legends. The cAMP extraction procedure was a modification of a method provided by the manufacturer (Diagnostic Products Corp.), originally described by Brown *et al.* [25]. The extraction efficiency of this procedure was >94% based upon the recovery data from samples spiked with [^{3}H]cAMP. In preliminary studies, using known amounts of cAMP, PCA extraction did not affect the ligand displacement in the competition binding assay. The sensitivity of the kit was from 0.11 to 27 pmol/tube. In most experiments, cAMP concentration in the samples ranged from 0.5 to 10 pmol/tube. However, for determination of unstimulated, basal levels of cAMP, a more sensitive method using acetylation of cAMP and subsequent radioimmunoassay (Amersham), which measured from 2 to 128 fmol/tube, was utilized.

In some experiments, the phosphodiesterase inhibitor Rolipram (Berlex Laboratories) was included to increase the accumulation of cAMP for measurements. In preliminary studies, to increase the sensitivity of the studies involving ligand-stimulated cAMP production (epinephrine, 10^{-6} M), cAMP catabolism was inhibited with various concentrations of Rolipram. Epinephrine-stimulated cAMP production was higher in the presence of Rolipram even at the lowest concentration: 0 μM

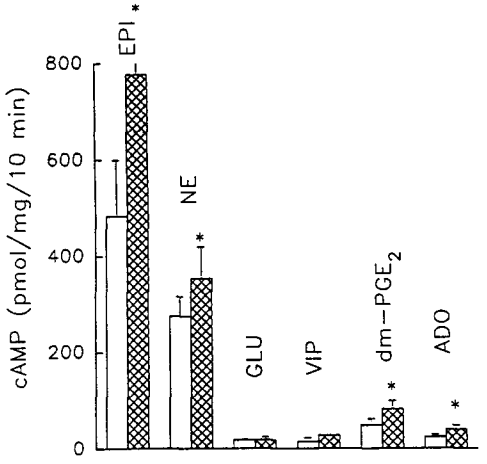

Fig. 1. Effects of different ligands on cAMP production in control and EtOH-treated cultured trophoblasts. Trophoblasts were treated with EtOH for 3 days as described in Materials and Methods. On the day of the experiment, cells were washed with isotonic buffer and treated with 10 μM Rolipram for 5 min. Trophoblasts were treated with epinephrine (EPI, 10^{-6} M), norepinephrine (NE, 10^{-6} M), glucagon (GLU, 10^{-6} M), vasoactive intestinal peptide (VIP, 10^{-6} M), 16,16'-dimethyl-prostaglandin E_2 (dm-PGE_2, 10^{-6} M), and adenosine (ADO, 5×10^{-5} M) in the presence of Rolipram. After 10 min, the reaction was terminated with PCA (0.5 M final concentration), and cAMP was determined using the binding protein displacement assay (Diagnostic Products Co.). Each experiment was carried out using 4 determinations. Data are means ± SEM from at least 3 placental preparations. Key: (*) $P < 0.05$ vs control. Control = open bars; EtOH-treated = cross-hatched bars.

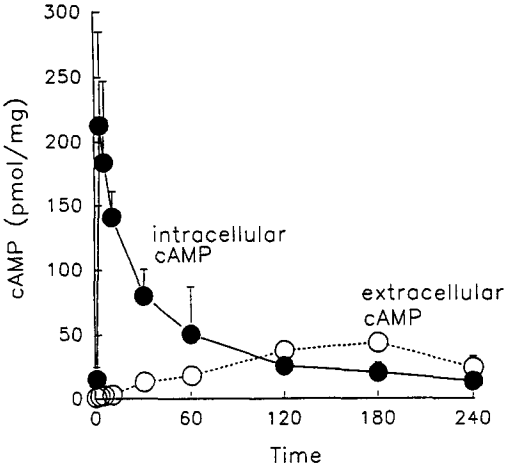

Fig. 2. Distribution of epinephrine-induced cAMP. Trophoblasts maintained in culture for 2 days were treated with epinephrine (10^{-6} M), and the reaction was terminated at indicated times by placing the cells on ice, rapidly removing the medium, and PCA extraction of the cell. cAMP concentrations in both the medium [extracellular (○)] and cell extracts [intracellular (●)] were determined. Values are means ± SEM, N = 3.

Rolipram = 228 ± 22; 2 μM = 293 ± 19; 10 μM = 313 ± 33; 50 μM = 298 ± 10 pmol/mg protein/10 min, $P < 0.05$. Therefore, when used, the Rolipram concentration was 10 μM.

Biochemical analyses. The hCG in the culture medium was determined using a radioimmunoassay kit (Ciba-Corning Diagnostics Corp.), which quantitates both the β subunit and intact hCG. Protein content of each culture well was determined by the method of Markwell *et al.* [26].

Data analyses. Each experimental cell preparation is denoted by (N). For each cell preparation, hormone or cAMP production was determined from 3–6 separate dishes (N) for a given condition and/or time point. The mean of such replicates was used as a single datum point for analysis, unless stated otherwise. All data are expressed as means ± SEM. Because of wide variations in cAMP production between different placental preparations, the Wilcoxon nonparametric signed rank sum test for paired samples was utilized to compare groups. Differences were considered significant for $P < 0.05$.

RESULTS

Effect of various ligands on cAMP production. To determine the effect of different hormones on cAMP production by control and EtOH-treated cultured trophoblasts, pharmacologic concentrations for each hormone were added for 10 min: 10^{-6} M of epinephrine, norepinephrine, dm-PGE_2, VIP, and glucagon. For adenosine, 5×10^{-5} M was used due to previously reported low activity in these cells [5]. At equimolar concentration of hormones, varied responses were observed with the following order of activity in control cells: epinephrine > norepinephrine > dm-PGE_2 > glucagon = VIP (Fig. 1). Although adenosine was used at a higher concentration than the other hormones, the cAMP response to adenosine was low. In EtOH-treated trophoblasts, epinephrine-, norepinephrine-, dm-PGE_2- or adenosine-stimulated cAMP production was enhanced compared with controls (Fig. 1). Therefore, it appears that EtOH induces a heterologous enhancement of ligand-stimulated cAMP production for at least some ligands.

Intra- and extracellular distribution of epinephrine-induced cAMP. Treatment of cultured trophoblasts with epinephrine (10^{-6} M) rapidly stimulated cAMP production (Fig. 2). Peak production occurred between 2.5 and 5 min after adding epinephrine and gradually returned to basal level over a 3- to 4-hr period. However, even after 60 min the intracellular cAMP concentration was higher than basal. In addition, a majority of cAMP remained intracellular up to 30–60 min. However, the extracellular cAMP increased gradually and exceeded the intracellular concentration by 2 hr. In most studies, the 10-min hormone treatment time point was used when intracellular cAMP accounted for approximately 99% of all cAMP produced.

EtOH exposure time and epinephrine-stimulated cAMP production. To determine the effect of EtOH treatment time on epinephrine-stimulated cAMP

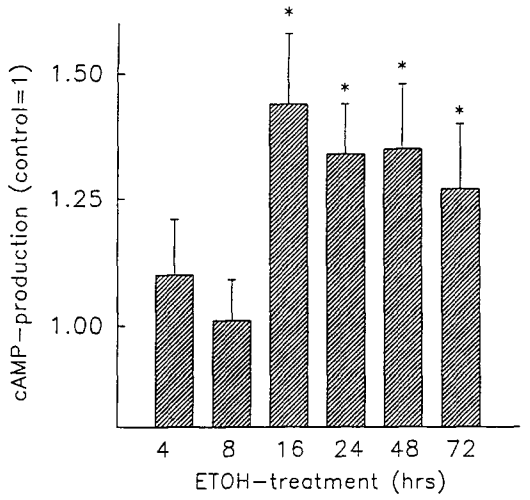

Fig. 3. EtOH exposure time and epinephrine-stimulated cAMP production. Starting at 24 hr of culture, trophoblasts were exposed to EtOH as described in Materials and Methods. At indicated times, trophoblasts were pretreated with Rolipram for 5 min, followed by Rolipram and epinephrine (10^{-5} M) for 10 min; then samples were extracted, and cAMP was determined. Data are for epinephrine-stimulated cAMP production in ethanol-treated relative to epinephrine-stimulated control cells of the same culture age. Values are means ± SEM, N = 3. Key: (*) $P < 0.05$ compared with paired controls.

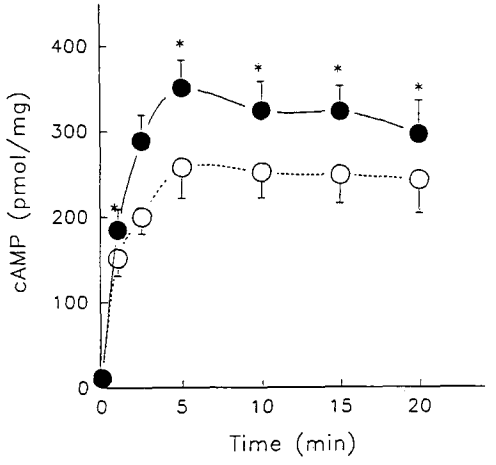

Fig. 4. Effect of EtOH on the time course of epinephrine-stimulated cAMP production. Trophoblasts were exposed to EtOH for 72 hr. Control and EtOH-exposed cells were treated with epinephrine (10^{-6} M) for 0, 1, 2.5, 5, 10, 15, and 20 min. Values are means ± SEM, N = 5. Key: (*) $P < 0.05$ compared with paired controls. Control = open circles; EtOH-treated = filled circles.

production, trophoblasts were exposed to EtOH for 4, 8, 16, 24, 48, and 72 hr. Enhancement of ligand (epinephrine) stimulated cAMP production was dependent upon the length of EtOH exposure (Fig. 3). A significant increase was observed beginning at 16 hr of exposure, and the response remained elevated through 72 hr. In these studies, there was a gradual increase in the medium EtOH concentration, which was equilibrated from EtOH vapor. In additional experiments, EtOH was added directly to the culture dish to maintain a constant level of EtOH throughout the treatment period. Again, the enhancement of hormone-stimulated cAMP production depended on the length of EtOH exposure; it was indistinguishable from the previous experiment (data not shown). Therefore, in cultured trophoblasts, the changes in ligand-stimulated cAMP production which are induced by EtOH require at least 16 hr of exposure to EtOH. In contrast, the basal resting cAMP levels were not affected by EtOH treatment even after 72 hr (control = 7.4 ± 2.1; EtOH-treated = 9.0 ± 1.3 pmol/mg protein).

Effect of EtOH on the time course for epinephrine-stimulated cAMP production. To determine the effect of EtOH exposure on epinephrine treatment time-dependent cAMP production, trophoblasts were exposed to EtOH for 72 hr. Control and EtOH-exposed trophoblasts were treated with epinephrine (10^{-6} M) for varying time periods up to 20 min. In both control and EtOH-exposed cells, epinephrine-stimulated cAMP production followed a similar pattern, with a plateau from 5 through 20 min (Fig. 4). However, the cAMP response was significantly higher in EtOH-exposed cells.

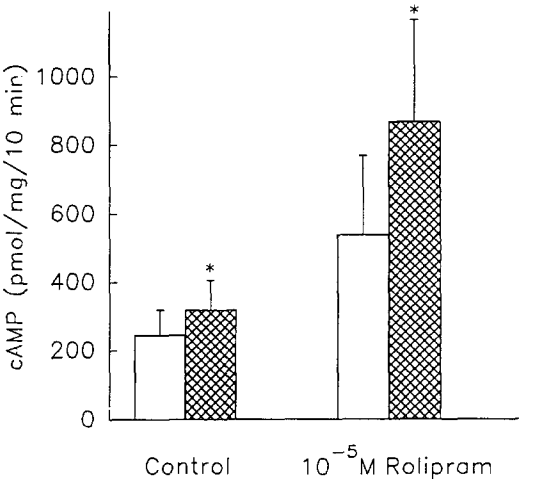

Fig. 5. Effect of phosphodiesterase inhibition during cAMP production in EtOH-treated trophoblasts. Trophoblasts were exposed to EtOH for 72 hr. Control and EtOH-exposed cells were treated with Rolipram for 10 min, and then epinephrine (10^{-6} M) for 10 min. The reaction was terminated and samples were processed to determine cAMP. Values are means ± SEM, N = 7. Key: (*) $P < 0.05$ compared with paired controls. Control = open bars; EtOH-treated = cross-hatched bars.

Effect of phosphodiesterase inhibition on EtOH-induced cAMP production. One mechanism by which EtOH might increase cAMP is altered cAMP

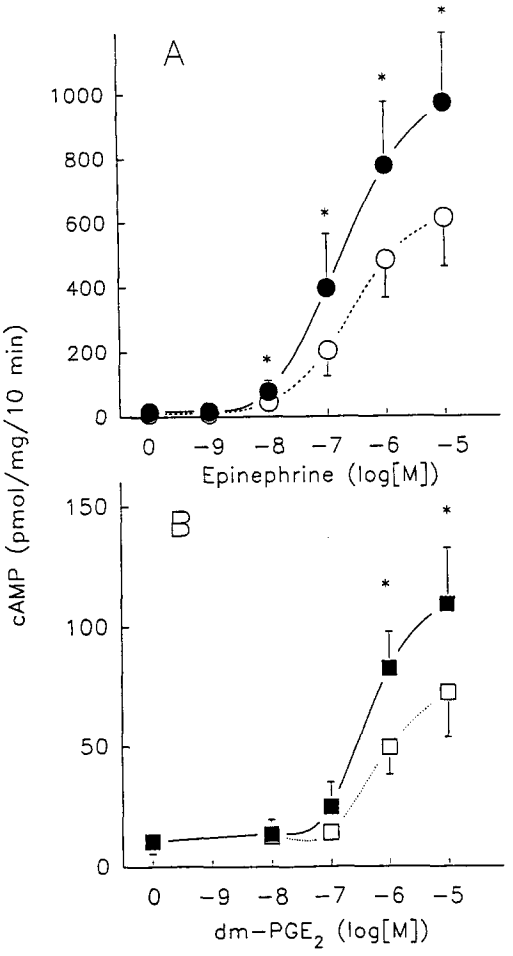

Fig. 6. Effect of EtOH on epinephrine (A) or dm-PGE_2 (B) concentration–response for cAMP production. Trophoblasts were exposed to EtOH for 72 hr. Control and EtOH-exposed cells were treated with Rolipram for 5 min, and then epinephrine (10^{-9}–10^{-5} M) or dm-PGE_2 (10^{-8}–10^{-5} M) for 10 min in the presence of Rolipram. Reactions were terminated and samples were processed to determine cAMP (N = 6 for epinephrine; N = 4 for dm-PGE_2). Values are means ± SEM. Key: (*) $P < 0.05$ compared with paired controls. Control = open symbols; EtOH-treated = filled symbols.

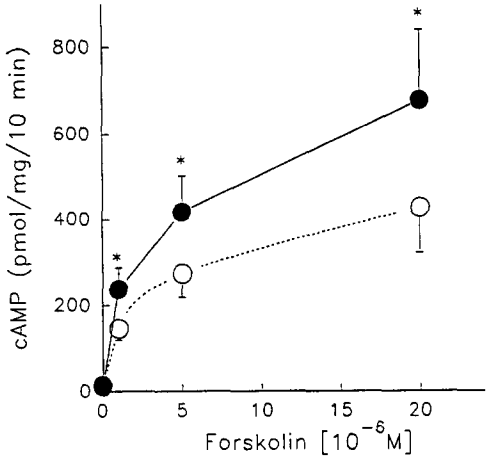

Fig. 7. Effect of EtOH treatment on forskolin-stimulated cAMP production. Trophoblasts were exposed to EtOH for 72 hr. Control and EtOH-exposed cells were treated with Rolipram for 5 min, and then Rolipram and forskolin (2×10^{-6}, 5×10^{-6}, 20×10^{-6} M) for 10 min. Reactions were terminated and samples were processed to determine cAMP. Values are means ± SEM, N = 6. Key: (*) $P < 0.05$ compared with paired controls. Control = open circles; EtOH-treated = filled circles.

catabolism. Therefore, epinephrine-stimulated cAMP production was determined in the presence and absence of 10 μM Rolipram, a potent inhibitor of phosphodiesterase (Fig. 5). The epinephrine-induced, EtOH-enhanced rise in cAMP was greater in Rolipram-treated cells (no Rolipram = 129%; Rolipram = 161% relative to control). Therefore, it appears that the EtOH-induced increase in ligand-stimulated cAMP production is not due to an inhibition of Rolipram-sensitive phosphodiesterase activity.

Effect of EtOH treatment on epinephrine or dm-PGE_2 concentration response for cAMP production. To determine whether EtOH-enhanced ligand-stimulated cAMP production is dependent upon the ligand concentration, EtOH-treated trophoblasts were exposed to different concentrations of epinephrine (10^{-9}–10^{-5} M) or dm-PGE_2 (10^{-8}–10^{-5} M) for 10 min. As shown in Fig. 6, EtOH treatment appeared to increase the capacity to respond to both ligands.

Effect of EtOH treatment on forskolin-stimulated cAMP production. To evaluate whether the EtOH effect was due to changes in intracellular signaling mechanisms, EtOH-treated cells were treated with forskolin, a direct activator of adenylyl cyclase. In control cells, forskolin-stimulated cAMP production was dependent upon forskolin concentration with more than 35-fold stimulation at 20 μM forskolin (Fig. 7). With EtOH treatment, there was increased cAMP production at all concentrations of forskolin (1–20 μM).

Effect of short-term EtOH exposure on epinephrine- or forskolin-induced cAMP production. To determine the effect of short-term exposure to EtOH on epinephrine-stimulated cAMP production, cultured trophoblasts were exposed to various concentrations of EtOH (60, 150, and 500 mg/dL) for 10 min. Subsequent stimulation with epinephrine (10^{-6} M) caused a rise in cAMP production, but this short-term EtOH treatment had no effect on epinephrine-stimulated cAMP production at any concentration (no EtOH = 374 ± 80; 60 mg EtOH/dL = 379 ± 70; 150 mg EtOH/dL = 326 ± 56; 500 mg EtOH/dL = 312 ± 35 pmol/mg protein/10 min). In addition, a concentration–response curve for epinephrine during short-term exposure to 300 mg EtOH/dL was determined to look for more subtle effects (Fig. 8A). Again, cAMP production was dependent upon the ligand concentration. EtOH pretreatment for 10 min followed by an additional 10 min during

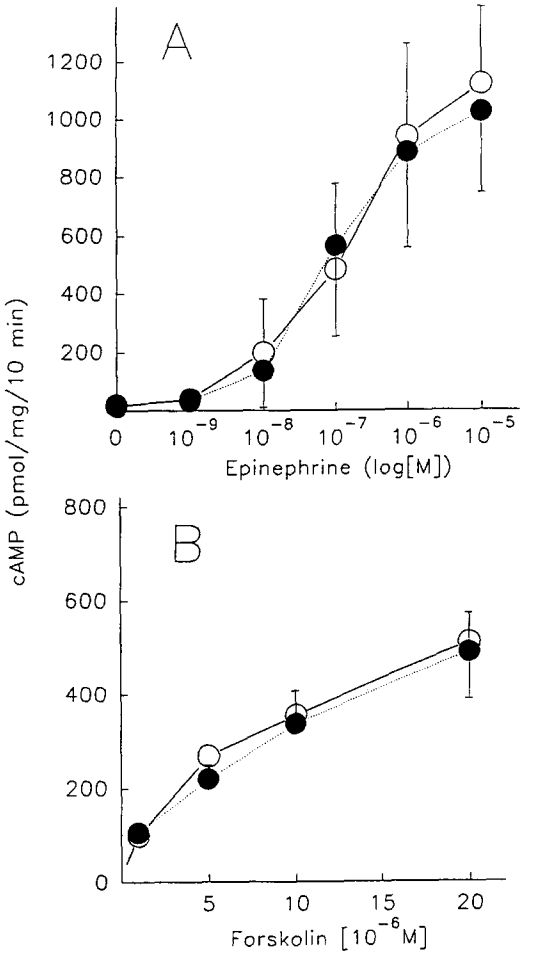

Fig. 8. Effect of short-term EtOH treatment on epinephrine- (A) or forskolin- (B) stimulated cAMP production. Cells were treated for 10 min with EtOH (300 mg/dL) in the presence of Rolipram (10 μM). Epinephrine (10^{-9}–10^{-5} M) (N = 5) or forskolin (10^{-6}–2×10^{-5} M) (N = 3) was added for 10 min. Reactions were terminated and samples processed to determine cAMP. Values are means ± SEM. Control = open circles; EtOH-treated = filled circles.

ligand treatment did not alter epinephrine-stimulated cAMP production. Similarly, short-term EtOH treatment had no effect on forskolin-stimulated cAMP production (Fig. 8B). These data suggest that a short-term EtOH treatment does not result in altered cAMP production in trophoblasts.

Effect of EtOH and various hormones on cAMP production in the JAR choriocarcinoma cell line. To explore the possibility of using an established cell line for studies of EtOH on ligand-induced cAMP production, JAR choriocarcinoma cells were treated with epinephrine, adenosine, dm-PGE_2 or VIP. In this cell line, only VIP had any effect on cAMP production (control = 8 ± 1; VIP-treated = 25 ± 6 pmol/mg protein/10 min). However, JAR cells did respond to forskolin, a direct activator of adenylyl cyclase, which resulted in a large production of cAMP (Table 1). Therefore, although the JAR cells had lost the ability to respond to various hormones, the adenylyl cyclase could be stimulated by forskolin. EtOH treatment up to 72 hr had no effect on forskolin-stimulated cAMP production in the JAR cells (Table 1). Therefore, the JAR choriocarcinoma cells are not a suitable model for studying EtOH-induced changes in ligand-stimulated cAMP production.

Table 1. Effect of 72-hr EtOH treatment on forskolin-stimulated cAMP production in JAR cells

Forskolin (μM)	cAMP production (pmol/mg/10 min)	
	Control	EtOH-treated
0	10.7 ± 2.3	7.3 ± 1.4
1	431.3 ± 34.7	365.3 ± 29.3
5	1088.3 ± 164.4	1127.0 ± 113.7

Cultured JAR choriocarcinoma cells were exposed to EtOH for 3 days as described in Materials and Methods. On the day of the experiment, JAR cells were treated with 10 μM Rolipram for 5 min prior to the addition of forskolin (0, 1 or 5 μM) for 10 min. The reaction was terminated and cAMP was determined. Values are means ± SEM, N = 3.

DISCUSSION

During pregnancy, the maintenance of normal placental function is critical to fetal growth and development. Alterations in normal placental physiology could be detrimental to the developing fetus. This and other laboratories have postulated that EtOH may alter placental function, contributing to EtOH-induced embryopathy [3, 4]. Feto-toxic effects of EtOH are dependent upon many variables. EtOH could have multiple sites of action including direct toxic effects on the fetus as well as indirect effects via altered maternal or placental physiology. This current report gives added insight into potential mechanisms for which EtOH may cause placental dysfunction: altered ligand-stimulated cAMP production.

In previous studies, experimental animal models involved either acute *in vitro* exposure to EtOH or chronic *in vivo* exposure followed by either *in vivo* or *in vitro* functional studies [27–31]. Unlike animal studies, acute *in vitro* exposure of human placenta to EtOH showed toxicity only at high pharmacologic concentrations [32, 33]. Until recently, no experimental model was available to examine the effect of longer term EtOH exposure on human placental cell function. Utilizing primary culture of purified human placental trophoblasts, we have demonstrated a model for studying both short and longer term effects of EtOH on the human placenta [5]. In that study, continuous exposure to EtOH for 2–4 days led to increased human chorionic gonadotropin and progesterone, but not human placental lactogen production. This change was associated with increased cAMP production by trophoblasts in response to adenosine. Our current data suggest

that enhanced adenylyl cyclase activation (either increased enzyme levels or altered G protein regulation) results from long-term EtOH treatment of cultured trophoblasts. This EtOH effect appears to apply to several ligands capable of stimulating cAMP production in trophoblasts, indicating heterologous sensitization.

EtOH-induced sensitization to ligand-stimulated cAMP production occurred only after 16 hr of exposure. This argues against a physical disturbance of the membranes by EtOH as the direct cause of altered membrane-associated function. However, compensatory changes in membrane structure might have been contributory. Nonetheless, EtOH treatment appears to increase the cell capacity for cAMP production in response to several ligands. This phenomenon does not appear to be due to inhibition of cAMP catabolism, as the EtOH effect persisted and was even more pronounced in the presence of Rolipram. It is unlikely that EtOH has an inhibitory effect on phosphodiesterase activity in trophoblasts, although direct measurements of phosphodiesterase levels were not performed.

EtOH-induced sensitization could also involve changes in the receptors, G proteins (G_i or G_s), or adenylyl cyclases. The fact that forskolin-stimulated cAMP production was also increased in EtOH-treated cells suggests that EtOH-induced changes probably involve events that are distal to ligand–receptor interaction. However, ligand receptor number/function was not measured directly. Since forskolin is a direct activator of the catalytic subunit of adenylyl cyclase, EtOH may have altered the concentration of the enzyme. On the other hand, forskolin activation of adenylyl cyclase is at least partially dependent upon the presence of G proteins [34]. Therefore, either the adenylyl cyclase enzyme or G protein mediated activation of adenylyl cyclase may be altered by EtOH treatment. Failure to observe an EtOH effect on glucagon or VIP stimulation would argue against a generalized increase in adenylyl cyclase levels, but the different isotypes of adenylyl cyclase that might be selectively affected by EtOH remain to be defined in the human placenta and could account for this differential response. Moreover, the human trophoblast response to glucagon and VIP is generally low to begin with, possibly affecting the technical ability to observe an effect of EtOH. Nevertheless, EtOH-induced sensitization appears to involve at least an altered function of G_s or adenylyl cyclase. Whether these changes are a result of a direct effect on these signal components is not known. Furthermore, EtOH effect may be mediated by other signal transduction pathways (cross-talk). In this regard, activation of protein kinase C by phorbol esters does up-regulate ligand-stimulated cAMP production in trophoblasts (unpublished data). Whether EtOH treatment directly or indirectly activates protein kinase C is under investigation. Further studies are necessary to determine the exact mechanisms involved in EtOH-induced sensitization of ligand-stimulated cAMP production.

Since primary trophoblast culture is a moderately labor intensive experimental model and cultures can rarely be maintained past 6–8 days, JAR choriocarcinoma cells were also tested for cAMP response to different ligands. Unlike the primary human trophoblast in culture, the JAR cell responds only to VIP, and then only modestly. However, the JAR cells did respond to forskolin. Moreover, EtOH treatment of JAR cells did not lead to changes in either VIP- or forskolin-stimulated cAMP production (data not shown). Therefore, the JAR cells may not be a good model for studying EtOH- and ligand-stimulated adenylyl cyclase activity. Rather, the response of these transformed cells to EtOH is different from that of normal cells, and there are numerous examples of cell type specific EtOH effects on the adenylyl cyclase pathway. In NG180-15 cells [18], chronic exposure to EtOH results in decreased ligand-stimulated adenylyl cyclase activity [18] as a result of down-regulation of the α subunit of the stimulatory guanine nucleotide binding regulatory protein ($G_s\alpha$) [35, 36]. However, in N18TG2 cells, EtOH treatment increases $G_i\alpha$ [36], also suggesting a cell-specific action. In addition, 8-week *in vivo* EtOH exposure approximately doubled glucagon-stimulated cAMP production in rat liver membrane [13]. Our findings add to the argument that the effect of EtOH on the adenylyl cyclase pathway varies with the tissue under study. It may be speculated that this is related to tissue-specific differences in relative amounts of adenylyl cyclase isotypes or G proteins.

In summary, these experiments indicated that EtOH enhances ligand-stimulated cAMP production in normal human placental trophoblasts. We suggest that this could lead to alterations in certain cAMP-dependent placental events. Since these signals are known to modulate cell function as well as cell differentiation and cell growth, EtOH-induced sensitization of the cAMP-dependent pathway may disrupt normal *in vivo* trophoblast function. Whether this effect also occurs at the fetal tissue level remains for further investigation.

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