Expression of Human Chorionic Gonadotropin (hCG)/Luteinizing Hormone Receptors and Regulation of the Cyclooxygenase-1 Gene by Exogenous hCG in Human Fetal Membranes

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ABSTRACT
The present study characterized hCG/LH receptors from messenger ribonucleic acid (mRNA) to protein and whether exogenous hCG can bind and regulate the expression of the cyclooxygenase-1 (COX-1) gene in human fetal membranes from term pregnancy.

Northern blotting showed that fetal membranes contain 6.0, 4.4, 2.4, and 1.4 kilobases of hCG/LH receptor mRNA transcripts. In situ hybridization revealed that amnion, chorion, and decidua contain receptor transcripts. Western immunoblotting and immunocytochemistry showed that amnion, chorion, and decidua contain an 80-kDa receptor protein. Ligand blotting demonstrated that the 80-kDa receptor protein in fetal membranes can bind [125I]hCG, and this binding was inhibited by excess unlabeled hCG. Treatment of fetal membranes with highly purified hCG resulted in a dose- and time-dependent increase in immunostaining of COX-1 protein. The expression of hCG was seen in all layers of fetal membranes. The treatment with hCG also resulted in an increase in steady state COX-1 mRNA levels. The action of hCG was prevented by cotreatment with H-89, an inhibitor of protein kinase A, but not by calphostin or lavendustin, which inhibit protein kinase C and tyrosine kinase, respectively.

In summary, human fetal membranes contain hCG receptor transcripts and receptor protein that can bind hCG and up-regulate the expression of COX-1 gene. (J Clin Endocrinol Metab 81: 1283-1288, 1996)

Materials and Methods

Materials
Full-length porcine hCG/LH receptor complementary DNA (cDNA) was obtained from Dr. Hugues Loosfelt of Hormones et Reproduction, Hospital de Bicetre, Kremlin Bicetre (Paris, France). The cDNA of COX-1 was obtained from Dr. Colin Funk at Vanderbilt University Medical Center (Nashville, TN). Polyclonal antibody raised against a synthetic N-terminal rat hCG/LH receptor peptide sequence of 15-38 ([anti-LHR-15-38]) and the corresponding synthetic peptide were obtained from Dr. Patrick Roche of the Mayo Clinic (Rochester, MN). Highly purified unlabeled hCG (CR 127; 14,900 IU/mg) was obtained from the National Hormone and Pituitary Program supported by the NIDDK, NICHD, and USDA (Rockville, MD). A monoclonal antibody to ovine seminal vesicle COX-1 was purchased from Cayman Chemical Co. (Ann Arbor, MI), and highly purified COX-1 protein from ovine seminal vesicles was obtained from Oxford Biomedical Research (Oxford, MI).

Collection of tissues
Twenty-four intact full thickness fetal membranes with adherent decidua were collected from elective cesarean section deliveries performed at term before the onset of labor for indications other than maternal or fetal distress. The membranes were not separated from each
other, and decidua was left with the membranes. In some experiments, the washed membranes were immediately frozen for later analysis by various blotting and histological techniques. For incubation studies, the membranes were placed in chilled Hank's buffer and immediately brought to the laboratory for further processing.

Northern blotting

This procedure was performed using 15 μg total RNA for hCG/LH receptors and 30 μg total RNA for COX-1 (8, 9). Prehybridizations were performed for 4 h at temperatures corresponding to subsequent hybridization temperatures. Hybridizations were performed with $1 \times 10^{6}$ cpm/mL of either 32P-labeled hCG/LH receptor cDNA for 16 h at 65°C or with COX-1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs for 16 h at 42°C. The blots were sequentially washed once for 10 min at 22°C with 2 X SSC containing 0.1% SDS, twice for 20 min each at 50°C with 1 X SSC containing 0.1% SDS, twice for 20 min each at 50°C with 0.1 X SSC containing 0.1% SDS, and finally once for 15 min at 50°C with 1 X SSC. The washed blots were exposed to Kodak x-ray film (Eastman Kodak, Rochester, NY) with intensifying screens for 5 days at -80°C.

In situ hybridization

This procedure was performed using $1 \times 10^6$ cpm/mL 32P-labeled antisense and sense riboprobes transcribed from full-length porcine hCG/LH receptor cDNA (9, 10). The hybridization with sense probe served as a control. Kodak NTB2 emulsion-coated slides were exposed in the dark at 4°C for a week, then developed and counterstained with hematoxylin.

Western immunoblotting

This procedure was performed using 25-μg aliquots of homogenate protein from fetal membranes, 1:1000 dilution of hCG/LH receptor and COX-1 antibodies, a 1:1000 dilution of horseradish peroxidase-labeled second antibody, and an enhanced chemiluminescence detection system (9, 11, 12). The receptor antibody preabsorbed with the receptor peptide was used for a control.

Ligand blotting

The procedure was performed with 30-μg aliquots of homogenate protein from fetal membranes (9, 13). The receptors were detected by using $1 \times 10^{6}$ cpm/mL $[^{32}P]$hCG in the presence and absence of 4 μg/mL unlabeled hCG. Unlabeled hCG was radioiodinated by the lactoperoxidase technique to a specific activity of about 80 μCi/μg (14).

Incubation of fetal membranes

Approximately 1-cm² segments of full thickness fetal membranes were cut and incubated at 37°C under 95% CO₂ and 5% O₂ in Ham's F-12-DMEM culture medium containing 10% calf serum. At the end of incubation, the membranes were extensively washed and processed for Western and Northern blotting. Further details are given in the figure legends.

Densitometry

The Bioquant Advanced System IV (Nashville, TN) was used for computerized quantitative video densitometry of Western and Northern blot data. The background values were measured, and corrections for them were made.

Repetition of experiments and statistics

All experiments were performed in duplicate or triplicate and repeated on two to six specimens from different patients. The values presented are the means and their standard errors. The data were analyzed by one-way ANOVA and the Tukey-Kramer post-hoc test.

Results

hCG/LH receptor transcripts in fetal membranes

Northern blotting shows that human fetal membranes (Fig. 1A, lane 1), like rat ovary (lane 3), which was used as a positive control tissue, contained multiple hCG/LH receptor transcripts of 6.0, 4.4, 2.4, and 1.4 kilobases (kb). As expected, rat ovary contained a greater abundance of receptor transcripts than fetal membranes. Rat kidney, used as a negative control tissue, did not show any detectable transcripts (lane 2).

In situ hybridization, performed to determine the localization of receptor transcripts in different layers of fetal membranes, showed that amnion (Fig. 2A), chorion (Fig. 2B), and decidua (Fig. 2C) contained hybridization signals with 32P-labeled antisense riboprobe. These hybridization signals were considerably reduced when 32P-labeled sense riboprobe was used for a procedural control (D).

hCG/LH receptor protein in fetal membranes

The presence of receptor transcripts led us to look for hCG/LH receptor protein in fetal membranes by Western immunoblotting. Figure 1B shows that fetal membranes (lane 1), like rat ovary (lane 3), contained an 80-kDa protein. The receptor protein was not detected in fetal membranes when the receptor antibody was preabsorbed with excess receptor peptide (lane 4) or in rat kidney when unabsorbed receptor antibody was used (lane 2). Again, as expected, rat ovary contained higher amounts of receptor protein than fetal membranes. The fetal membranes also contained a minor 50-kDa protein (data not shown).

Immunocytochemistry to determine the presence of receptor protein in different layers of fetal membranes showed that amnion, chorion, and decidua contained receptor immunostaining (Fig. 2, E and F). The receptor immunostaining dramatically decreased in all membrane layers after preabsorption of the antibody with excess receptor peptide (Fig. 2G).

![Fig. 1. Northern (A), Western (B), and ligand (C) blotting for hCG/LH receptors in human fetal membranes. A: Lane 1, Fetal membranes; lane 2, rat kidney as a negative control tissue; lane 3, rat ovary as a positive control tissue. B: Lane 1, Fetal membranes; lane 2, rat kidney; lane 3, rat ovary; lane 4, antibody preabsorption control for fetal membranes. C: Lanes 1 and 2, Fetal membranes; lane 3, rat ovary; lane 4, rat kidney. $[^{32}P]$hCG is present in all lanes, and excess unlabeled hCG is also present in lane 2.](https://academic.oup.com/jcem/article-abstract/81/3/1283/TOTH/2649753)
**Fig. 2.** *In situ* hybridization (A–D) and immunocytochemistry (E–G) for hCG/LH receptors in human fetal membranes. A and D, Amnion; B, chorion; C, decidua; E and F, low and high magnification pictures of fetal membranes, respectively; D, an *in situ* hybridization control with 32P-labeled sense probe; G, an immunocytochemistry control with preabsorbed antibody. *Arrows* point out amnion; the *large arrowheads* point out chorion, and *small arrowheads* point out decidua. Magnification: A–D, ×1500; E, ×150; F and G, ×600.
Ligand blotting demonstrates that an 80-kDa protein in fetal membranes (Fig. 1C, lane 1), just as in ovary (lane 3), can bind $^{125}\text{I}hCG$. As expected, rat ovary bound more $^{125}\text{I}hCG$ than fetal membranes, and the membrane binding of $^{125}\text{I}hCG$ was considerably reduced in the presence of excess unlabeled hCG (lane 2). The binding of $^{125}\text{I}hCG$ was not detectable in rat kidney (lane 4). The minor 50-kDa protein found in fetal membranes did not bind $^{125}\text{I}hCG$ (data not shown).

**Dose and time dependency of effect of hCG on COX-1 protein in fetal membranes**

Western immunoblotting detected a 72-kDa COX-1 protein that responded to hCG treatment (Fig. 3). As shown in Fig. 3, although 0.01 ng/mL hCG had no effect, 0.1 ng/mL and higher concentrations of hCG significantly increased COX-1 protein levels compared to the control values. The extent of increase remained about the same at a wide hCG concentration range of 1–10,000 ng/mL.

The COX-1 protein also responded to hCG treatment in a time-dependent manner (Fig. 4). Although treatment with 10 ng/mL hCG for 1 h had no effect, 2 h of treatment significantly increased COX-1 protein levels compared to the control values. At 4 h, hCG had a modest stimulatory effect, which disappeared after incubation for 6, 16, and 24 h.

**Signaling in hCG action**

Figure 5 shows, as before, that treatment of fetal membranes with 10 ng/mL hCG for 4 h resulted in a significant increase in COX-1 protein. The presence of H-89, which inhibits protein kinase A, but not calphostin or lavendustin, which inhibit protein kinase C and tyrosine kinase, respectively, prevented the hCG-induced increase in COX-1 protein.

**Effect of hCG on steady state COX-1 mRNA levels**

The stimulatory effect of hCG on COX-1 protein led us to investigate the effect of hCG on COX-1 mRNA levels. Figure 6 shows that fetal membranes contain 4.1- and 2.8-kb COX-1 transcripts. Levels of both transcripts significantly increased after treatment for 4 h with 100 ng/mL hCG compared to control values (Fig. 6). The effect was specific, as hCG had no influence on the mRNA levels of a housekeeping gene, GAPDH. This allowed us to express the densitometric values as ratios of COX-1/GAPDH.

**Discussion**

The present study extended our earlier immunocytochemical data by systematically examining the presence of receptor mRNA transcripts to receptor protein that can bind $^{125}\text{I}hCG$ and regulate a function in reflected fetal membranes.

First, Northern blotting and in situ hybridization demonstrated the presence of multiple receptor transcripts in amnion, chorion, and decidua. Western blotting and immuno-
FIG. 6. Effect of hCG on steady state mRNA COX-1 levels in human fetal membranes. The membranes were incubated at 37 C for 4 h in the presence or absence of 100 ng/mL hCG treatment. Densitometric values as a ratio of COX-1/GAPDH, with the corresponding Northern blot in the inset, are presented. An asterisk indicates a difference compared to the control (P < 0.01).

cytochemistry demonstrated the presence of an 80-kDa receptor protein in all layers of fetal membranes that contained the receptor transcripts. Ligand blotting demonstrated that 80-kDa protein can bind [125I]hCG. The receptor levels in fetal membranes were lower than those in rat ovary, which was used as a positive control tissue.

COX-1 is one of the two enzymes involved in converting free arachidonic acid into PG endoperoxide H, which is further converted to different eicosanoids by corresponding isomerases (15). It is generally believed that COX-1 is constitutively expressed, whereas COX-2 is a regulated enzyme in various tissues (15). Fetal membranes contain both of these enzymes (16-18). Our previous study has shown that treatment of primary cultures of amnion cells as well as immortalized amniotic epithelial WISH cells with hCG resulted in an increase in immunostaining for COX-1 (7). These findings led us to focus on COX-1 in determining the functional relevance of hCG/LH receptor proteins in full thickness membranes. The results showed that treatment with hCG resulted in a dose- and time-dependent increase in COX-1 protein levels. The disappearance of hCG response after 4 h of incubation is not due to cell death, as fetal membranes continue to maintain high levels of COX-1 protein for up to 24 h. Immunocytochemical analysis revealed that the hCG-induced increase in COX-1 protein occurred in amnion, chorion, and decidua (data not shown). The increase in COX-1 protein appears to be a corresponding increase in steady state COX-1 mRNA levels.

As COX-1 is generally believed to be an unregulated enzyme (15), the present data demonstrating that hCG can regulate COX-1 are somewhat surprising. Our preliminary data showed that hCG treatment can also increase COX-2 with different response characteristics from COX-1 (Toth, P., X. Li, Z. M. Lei, and Ch. V. Rao, unpublished data). The concentrations of hCG that increased COX-1 and COX-2 also modestly increased immunostaining for PGE. (Toth, P., X. Li, Z. M. Lei, and Ch. V. Rao, unpublished data), suggesting that the increased COXs are functional. In agreement with a recent study (19), our findings support the idea that COX-1 and COX-2 have different functions, use different pools of endogenous lipids, and operate as two independent systems in the biosynthesis of eicosanoids.

The eicosanoids produced by fetal membranes are generally considered to play a role in the initiation of labor (20, 21). The process of hCG, a pregnancy-maintaining hormone, stimulating COX-1, thus potentially increasing the formation of eicosanoids, which leads to the initiation of labor, is not consistent with the above concept. Therefore, it is possible that hCG treatment may increase another downstream enzyme in the COX pathway, such as PGI synthase, leading to the formation of PGL, which can inhibit myometrial contractions. In fact, hCG treatment of amnion and WISH cells results in an increase in PGI synthase (7). It could be that the eicosanoids produced by fetal membranes may regulate the functions of the membranes themselves rather than initiating the labor. Several recent reports suggested that PGE produced by amnion is mostly degraded before it can be converted to PGF by chorion and decidua; eicosanoids produced by fetal membranes are an aftereffect of labor, and finally, fetal membranes inhibit uterine contractions (22-28).

In summary, human fetal membranes contain hCG receptor transcripts and protein that can bind hCG and up-regulate the expression of the COX-1 gene. These findings suggest that hCG may directly regulate the functions of fetal membranes that are consistent with maintaining pregnancy.

References

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