HOXA10 regulates endometrial GABA$_A$ receptor expression and membrane translocation

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Sadeghi H, Taylor HS. HOXA10 regulates endometrial GABA$_A$ receptor expression and membrane translocation. Am J Physiol Endocrinol Metab 298: E889–E893, 2010. First published January 26, 2010; doi:10.1152/ajpendo.00577.2009.—Expression of the GABA$_A$ receptor has been described previously in the human endometrium in both luminal epithelium and stroma. Its expression is increased during decidualization in rodents and in the implantation window of human endometrium. Here we localized GABA$_A$ subunit receptor protein in human endometrium and identified regulators of gene expression and activation. GABA$_A$ receptor was localized to the cell surface, and expression increased during the window of embryo implantation in human endometrium. The well-differentiated human endometrial adenocarcinoma cell line Ishikawa was treated with progesterone and transfected with pcDNA-HOXA10, HOXA10 siRNA, or respective controls. GABA$_A$ receptor mRNA expression was evaluated by real-time RT-PCR. Protein expression and localization were evaluated using immunofluorescence. GABA$_A$ receptor mRNA expression was increased significantly after either progesterone treatment or HOXA10 transfection. Coinadministration of progesterone along with HOXA10 transfection had no additional effect on the expression of GABA$_A$ receptor mRNA over either agent alone. Blocking HOXA10 expression with siRNA prevented progesterone-induced GABA$_A$ receptor mRNA expression. Additionally, either HOXA10 or progesterone independently caused increased translocation of the GABA receptor from the cytoplasm to the cell membrane. Translocation in response to progesterone was blocked with HOXA10 siRNA. Progesterone-induced GABA$_A$ subunit receptor expression is likely mediated indirectly through progesterone’s regulation of HOXA10 expression. Modification of subtype composition and translocation of the GABA receptor ion channel likely modulate endometrial receptivity. Whereas HOXA10 typically enhances the expression of progesterone-responsive genes, here HOXA10 expression leads to production of a less progestin-responsive GABA receptor subtype, likely buffering the effects of luteal phase progesterone on GABA receptor activity.

GABA$_A$ receptors are fast-acting, ligand-gated chloride ion channels that are allosterically regulated by compounds such as anxiolytics, barbiturates, anesthetics, and neurosteroids, including reduced derivatives of progesterone such as allopregnanolone. (7, 19, 25, 44) Several GABA$_A$ subunits have been cloned, many with several isoforms (1, 11, 31). They are also present in peripheral tissues outside of the brain, including the female reproductive tract, where the subunit composition of the GABA$_A$ receptor fluctuates in the rat uterus throughout pregnancy. (2, 12–14, 30) Isoform selection affects the sensitivity and responsiveness of the GABA$_A$ receptor to progesterone metabolites, which may help regulate parturition. (17)

Specifically, the GABA$_A$ receptor is expressed in the brain and in several noneuronal tissues, including the human endometrium and rat uterus (21). The subunit concentration has been found to vary throughout the cycle and gestation (23, 33). Receptor expression is upregulated during the window of implantation in well-characterized human endometrial biopsies. (23) The receptor’s phenotypic localization and timing of expression have been suggested to play an important role in the generation of receptive endometrium.

Homeobox genes are a family of highly evolutionarily conserved transcriptional regulators termed HOX in humans and Hox in mice. (29) Several HOX/Hox genes regulate endometrial growth and development. Specifically, homeobox A10 (HOXA10) is a homeobox gene that is expressed in the human endometrial glands and stroma throughout the menstrual cycle (40). Estrogen and progesterone regulate endometrial expression of HOXA10. HOXA10 expression rises dramatically at the time of implantation, suggesting a role for HOXA10 in this process in humans. It is essential for fertility in mice. Targeted disruption of the Hoxa10 gene renders mice infertile due to defective endometrial receptivity and failed implantation. (4, 37) Hoxa10 antisense yields decreased Hoxa10 expression, number of implantation sites, and litter size in murine uteri. Conversely, overexpression of Hoxa10 increases litter size (3). HOXA10 is likely to mediate a number of sex steroid effects by activation or repression of target genes, potentially including those encoding for the GABA$_A$ receptors.

Here we investigated the regulation of GABA$_A$ receptor subunit expression and its functional activation through membrane translocation. Both HOXA10 and GABA$_A$ receptor subunit receptors are expressed in the endometrium at the time of embryonic implantation (21). They are both regulated and/or modulated by the sex steroid progesterone (45). A recent microarray-based study from our laboratory identified the GABA$_A$ receptor as a potential Hoxa10 target gene in the murine endometrium (45). Here we demonstrate that not only.

$\gamma$-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain. Its effects are mediated via interactions with integral membrane proteins, the GABA receptors. These receptors are classified according to their pharmacological properties and mechanism of signal transduction. (7, 38, 46)

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was GABA_A_π subunit gene expression regulated by HOXA10, its membrane translocation was as well.

**MATERIALS AND METHODS**

**Cell culture.** Ishikawa is a well-differentiated endometrial adenocarcinoma cell line that has previously been used to model human endometrial epithelial cells. Ishikawa cells express progesterone receptors and other markers of endometrial function (5, 20, 22, 26–28, 36, 39). HOX gene expression has previously been well characterized in these cells (10, 35, 42, 43). Ishikawa cells were maintained in 25-cm² flasks, using Dulbecco’s minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere (5% CO₂ in air) and allowed to reach confluency. Cells were passaged by standard methods of trypsinization and plated on both glass microscope slides for immunofluorescence studies and on six-well plates for RNA analysis until they reached 75–80% confluence. The cells used for RNA extraction were treated with 10⁻⁶ M progesterone for 1 h. The cells used for immunofluorescence were treated with 10⁻⁶ M progesterone for 24 h. Each experiment was performed in triplicate and repeated four times.

**Transfection.** Preconfluent (75–80%) Ishikawa cells on glass slides were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with 1.6 µg of either pcDNA-HOXA10 3.1 or empty pcDNA3.1. Preconfluent (75–80%) Ishikawa cells in six-well plates were similarly transfected with 4 µg of either pcDNA-HOXA10 3.1 or empty pcDNA3.1, siRNA control, or 20 µM HOXA10 siRNA. pcDNA-HOXA10 3.1 expresses HOXA10 constitutively at high levels, as described previously, whereas pcDNA3.1 served as the respective control (10, 32, 35, 42, 43). HOXA10 siRNA was synthesized by siGENOME duplex (Dharmacon, Lafayette, CO). An unrelated siRNA served as a control. The cells were then washed with cold phosphate-buffered saline after 24 h and treated with serum-free, phenol red-free medium for 24 h. The cells were then treated with either 10⁻⁶ M progesterone or vehicle control for either 1 or 24 h for RNA or immunofluorescence studies, respectively.

**Real-time RT-PCR.** RNA from cells was isolated using Trizol (Life Technologies, Carlsbad, CA) pursuant to the manufacturer’s guidelines. One microgram of total RNA was reverse transcribed in 20 µl of reaction mixture containing 10 mM each of dATP, dGTP, dCTP, and dTTP, 20 pmol oligo(dT), 40 µM CdT30 ribonuclease inhibitor, 10 µM/µl avian myeloblastosis virus-reverse transcriptase, and 10 µM/µl avian myeloblastosis reverse transcriptase buffer for 30 min at 61°C. The GABA_A_π receptor intron-spanning primers were selected, using the primer selection program Primer3 developed by the Whitehead Institute for Biomedical Research. Quantitative real-time RT-PCR was performed using the Light cycle SYBR Green RT-PCR kit from Roche. The Light Cycler monitored the increasing fluorescence of PCR products during amplification. The samples were quantification adjusted to the quantitative expression of β-actin from the same samples. Melting curve analysis was conducted to determine the specificity of the amplified products and to ensure the absence of primer-dimer formation. All products obtained yielded the predicted melting temperature. Construction of a standard curve allowed quantification. At least three independent experiments were conducted. Paired and unpaired t-tests were applied as appropriate. Data are presented as means ± SE. A value of P < 0.05 was considered significant.

**Immunocytochemistry.** Tissue was obtained by endometrial biopsy from 10 normal reproductive-age women at the time of tubal ligation under a protocol that was approved by the Yale University School of Medicine Human Investigations Committee. Samples were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized by a 50–50% mixture of ethanol-acetone for 10 min followed by 0.05% Triton X-100 in phosphate-buffered saline (PBS) for 2 min at room temperature. They were then washed with PBS with 2% Tween. Non-specific binding sites were then blocked by incubation with 0.5% bovine serum albumin (BSA) in PBS for 15 min. Samples were then incubated overnight at 4°C with the primary antibodies [GABA_A_π receptor (sc-21338) from Santa Cruz Biotechnology (Santa Cruz, CA) and cytokeratin 5/18 (VP-C418) from Vector Laboratories (Burlingame, CA)]. This step was omitted in the negative control samples. The next day, the cells were incubated with the appropriate fluorescence-conjugated secondary antibodies in PBS-0.5% BSA [goat α-rabbit IgG (FI-1000) and horse α-mouse (TI-2000); Vector Laboratories] for 90 min at room temperature, washed again, and DNA stained with TO-PRO-3 [(1:10,000 for 10 min) and mounted with PBS-90% glycerol-1% p-phenylenediamine] or Dapi Vectashield with 4’,6-diamidino-2-phenylindole (Vector laboratories, Burlington, CA). Samples were then examined by confocal laser-scanning microscopy.

**Confocal laser-scanning microscopy.** Confocal microscopy was performed using a Zeiss LSM 510NLO/Multiphoton microscope. This microscope uses an argon laser and two HeNe lasers for excitation (at 458, 477, 488, 514, 543, and 633 nm) and also has three detection channels. Optical tomography was performed at 0.5-µm intervals using a 40- and 60-fold oil immersion objective. All observations were made on single Ishikawa cells in monolayers. Treated slides were divided into four quadrants. Each quadrant was evaluated by counting the number of cells with peripheral localization of GABA_A_π receptors, as evident by immunofluorescent staining per high-power field. Statistical significance was measured using Student t-tests.

**RESULTS**

**GABA π subunit expression is increased at the window of embryo implantation in human endometrium.** Immunohistochemistry was used to identify the timing of GABA_A_π subunit expression in human endometrium through the menstrual cycle. The subunit was expressed throughout the menstrual cycle in both epithelial and stromal cells. Expression was increased in the window of embryo implantation in human endometrium, as demonstrated in Fig. 1. The average number of endometrial cells expressing high levels of GABA_A_π subunit protein was 2% in the proliferative phase; the percentage increased to 93%...
in the midluteal phase ($P < 0.006$). Increased expression corresponded to the known time of acquisition of endometrial maturation to allow embryo implantation.

**Progesterone induces $\pi$-subunit expression in endometrial cells through a HOXA10-mediated pathway.** A significant increase in $\pi$-subunit receptor mRNA levels was seen in endometrial cells transfected with HOXA10 and treated with progesterone or the combination of the two compared with corresponding control vector-transfected or vehicle-treated samples, respectively (Fig. 2). $\pi$-Subunit receptor mRNA level was increased approximately threefold after HOXA10 transfection upon normalization with $\beta$-actin ($P = 0.001$). Similarly, subunit mRNA was increased approximately threefold after progesterone treatment ($P = 0.022$) and threefold after coadministration of both progesterone and HOXA10 ($P = 0.014$). No additive effect was observed in $\pi$-subunit receptor mRNA levels when progesterone was added to the HOXA10-transfected cells ($P = 0.294$) compared with progesterone-only-treated cells, suggesting a common signal transduction pathway.

To address the significance of the HOXA10-mediated positive regulation of the GABA receptor, cellular levels of HOXA10 were inhibited by an siRNA knockdown construct in Ishikawa cells. Cells were treated with HOXA10 siRNA for 24 h. This resulted in significant inhibition of mRNA levels of the $\pi$-subunit compared with controls. Cells treated with nonsilencing siRNA constructs showed no significant change in the receptor levels. $\pi$-Subunit receptor levels were decreased from basal levels when cells were transfected with HOXA10 siRNA constructs ($P = 0.009$). Progesterone treatment of cells along with transfection of HOXA10 siRNA also resulted in significantly decreased induction of $\pi$-subunit expression in Ishikawa cells, indicating that the effect of progesterone requires HOXA10.

**Progesterone induction of HOXA10 expression leads to membrane translocation of the GABA$\alpha_3$ $\pi$ receptor.** To assess the localization pattern of GABA$\alpha_3$ $\pi$ receptors within the cells, immunocytochemical analysis was performed on the Ishikawa cells and assessed by confocal microscopy. A significantly increased number of cells displayed peripheral localization of $\pi$-subunit receptor expression when cells were treated with progesterone compared with corresponding vehicle-treated cells (Fig. 3, A and B). Blockade of HOXA10 expression with siRNA resulted in elimination or significant reduction of GABA$\alpha_3$ receptor membrane translocation even in the presence of progesterone (Fig. 3C). Cells transfected with HOXA10 also demonstrated enhanced membrane translocation of the receptor in the absence of progesterone (Fig. 4). Quantification of the number of cells showing membrane translocation is displayed in Fig. 5. Cells treated with progesterone ($P = 0.002$), transfected with HOXA10 ($P = 0.001$), or treated with the combination of the two ($P = 0.008$) each showed significant membrane translocation compared with vehicle or vector-
transfected cells. Similarly, HOXA10 siRNA blocked the progesterone-mediated membrane translocation.

DISCUSSION

In addition to its role as a neurotransmitter, GABA may perform multiple functions in nonneuronal tissues. GABAA receptor activity depends on a number of variables; these include subunit arrangement and isoform selection, concentration, and timing of expression as well as localization to the plasma membrane. The human endometrium expresses the GABAA receptor (16, 24). The expression of this receptor is known to be modulated by progesterone and its metabolite allopregnanolone (16, 17, 24). The receptor is upregulated during the window of implantation in human endometrium (1, 11, 31) and is thought to play a role in endometrial receptivity. The expression of this receptor, as well as its membrane translocation, is likely to be involved in initiating and maintaining a receptive endometrium.

HOX genes encode highly evolutionarily conserved transcription factors that have evolved to serve an essential role in endometrial receptivity in mammals (8, 15). Endometrial HOXA10 expression fluctuates with the menstrual cycle, which is regulated by estradiol and progesterone levels. This results in an upregulation of the gene in the midluteal phase, a period corresponding to the time of implantation (36, 40). Hoxa10 expression is necessary for endometrial receptivity to embryo implantation in mice (3, 4, 37). Similarly, women with low implantation rates demonstrate diminished endometrial HOXA10 expression (6, 9, 34, 41).

There are no previous reports identifying regulators of GABAA receptor expression or activation. Here we established that HOXA10 regulates GABAA receptor mRNA expression and, surprisingly, also regulates its membrane translocation. HOX genes encode transcriptional regulators that typically bind regulatory elements of target genes and influence RNA synthesis. Due to the rapid induction of expression, it is likely that HOXA10 directly regulates the expression of GABA receptor mRNA. The receptor membrane localization occurs only after a delay of several hours; therefore, it is likely that this function of Hoxa10 is indirect. HOXA10 likely induces the expression of proteins involved in translocation to the plasma membrane. Future efforts will focus on delineating target chaperone proteins necessary for protein mobilization.

The function of GABA receptors in the uterus is still incompletely characterized. In the central nervous system, allopregnanolone modulates receptor activity and neuronal inhibition by altering the frequency and duration of GABA channel opening. The assembly of the γ-subunit into the GABA receptor reduces sensitivity to allopregnanolone. Suppression of GABA activity in the uterus is likely beneficial, since enhanced gabageneric tone is associated with contractions and labor. Therefore, subunit selection may enable the tolerance of the dramatic increase in progesterone and allopregnanolone in the luteal phase. Reduced sensitivity to allopregnanolone may facilitate implantation by attenuating GABA signaling and inducing quiescence at the time of implantation.

Although progesterone action is generally considered to be essential for endometrial differentiation and endometrial receptivity, not all of the myriad effects of progesterone necessarily promote implantation. HOXA10 is known to enhance progesterone action on the expression of many genes. However, here it likely buffers the effects of increased progesterone on this signal transduction pathway. We identify a novel role for HOXA10 as both a positive and negative modulator of progesterone responsiveness in the endometrium.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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