Estradiol rapidly inhibits soluble guanylyl cyclase expression in rat uterus

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Previous reports that investigated the regulation of the NO/soluble guanylyl cyclase (sGC)/cGMP pathway by estrogenic compounds have focused primarily on the levels of NO, NO-producing enzymes, and cGMP in various tissues. In this study, we demonstrate that 17β-estradiol (E2) regulates the α1 and β1 subunits of the NO receptor, sGC, at the mRNA and protein levels in rat uterus. Using real-time quantitative PCR, we found that within 1 h of in vivo E2 administration to rats, sGC mRNA levels begin to diminish. After 3 h, there is a maximal diminution of sGC mRNA expression (sGC α1 10% and sGC β1 33% of untreated). This effect was blocked by the estrogen receptor antagonist, ICI 182,780, indicating that estrogen receptor is required. The effect of E2 also was observed in vitro with incubations of uterine tissue, indicating that the response does not depend on the secondary release of other hormones or factors from other tissues. Puromycin did not block the effect, suggesting the effects occur because of preexisting factors in uterine tissues and do not require new protein synthesis. Using immunoblot analysis, we found that sGC protein levels also were reduced by E2 over a similar time course as the sGC mRNA. We conclude that sGC plays a vital role in the NO/sGC/cGMP regulatory pathway during conditions of elevated estrogen levels in the rat uterus as a result of the reduction of sGC expression.

The steroid hormone 17β-estradiol (E2) exhibits dramatic effects in the uterus with respect to hyperemia, gene expression, and proliferation of the endometrium. It is clear that there is a wide array of changes in the expression of multiple genes and biochemical processes within the uterus after E2 exposure. However, as a whole, it is unclear how these responses mediate the hyperemic and subsequent proliferative response of the uterine tissue.

To understand how these processes are regulated is essential, because normal physiological, pathologic, and pharmacologic estrogenic responses are common. For instance, the levels of estrogen are tightly regulated and mediate uterine quiescence and initiation of labor during pregnancy (1). In addition, estrogen levels fluctuate during the estrous cycle of mammals and the menstrual cycle of primates, which mediate the endometrial growth cycle (2). Postmenopausal women frequently are administered estrogen supplements in hormone replacement therapy regimens to prevent the loss of bone mass associated with menopause. Furthermore, use of the anti-estrogen, tamoxifen, for treating breast cancer results in paradoxical estrogenic effects in the uterus (3). Finally, both men and women are exposed to low levels of phyto-estrogens on a daily basis, dependent on dietary and environmental intake (4).

Reports indicate that estrogens regulate the NO/soluble guanylyl cyclase (sGC)/cGMP cell signaling pathway and the NO receptor, sGC, at the mRNA and protein levels in response to E2 in the uterus. In this report, we used immature rats to determine whether E2 regulates the mRNA and protein levels of the sGC subunits. We report that E2 has a rapid and profound effect on uterine sGC α1 and β1 isoforms in this model system, which is well characterized to study estrogenic regulation of gene expression in vivo.

Materials and Methods

Animals and Treatments. Immature female Sprague–Dawley rats (21 days old, 40–45 g; Sasco, Omaha, NE) were either non-ovariectomized, purchased ovariectomized, or ovariectomized on arrival; and ovariectomized animals were allowed to recover 4 days before treatments. Animals were given tap water and fed Purina rat chow (no. 5001) ad libitum, with a 12:12 h light/dark cycle. All procedures were approved by the University of Texas–Houston Health Science Center Animal Welfare Committee and are in accordance with the guidelines of the National Institutes of Health.

Animals were injected s.c. (as described previously) in the periscapular region with various hormones (23). Unless other-
wise indicated (dose–response), the dose of E2 was 40 µg/kg body weight, whereas the doses of other hormones were as follows: dexamethasone, 600 µg/kg; 5α-dihydrotestosterone, 400 µg/kg; and progesterone, 40 mg/kg (Sigma). For other experiments, animals were treated i.p. (as described previously) with either ICI 182,780 (2 mg/kg; Tocris Neuramin, Bristol, U.K.) or puromycin (100 mg/kg; Sigma) 30 min before the administration of 40 µg/kg E2 (23, 24).

**Determination of Uterine sGC α1 and β1 mRNA Levels in Vitro.** Animals were killed and uterine tissues were immediately removed and placed into glass vials containing 1 ml of pregressed (95% O2/5% CO2 at 37°C) phenol red-free DMEM/F-12 medium (GIBCO; supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin) either with or without 10 nM E2. All vials then were gassed with 95% O2/5% CO2, sealed, and incubated for 3 h in a 37°C shaking water bath.

**RNA Preparations.** Tissues were removed from killed animals or glass vials and immediately homogenized in Ultra Spec RNA Isolation Solution (Biotecx Laboratories, Houston) to obtain total RNA preparations. After isolation, total RNA from tissues was spectrophotometrically quantified at 260 nm. For Northern analysis, total RNA from three individual animals was pooled and subjected to poly(A)+ mRNA purification by using a poly(A)+ purification kit (DynaL, Great Neck, NY).

**Preparation of Tissue Homogenates for Immunoblot Analysis.** Tissues were removed from killed, nonovariectomized animals and placed immediately into liquid nitrogen and subsequently stored at −80°C until used (not more than 1 wk). Tissues were homogenized in buffer containing 50 mM triethanolamine (pH 7.4), 50 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, 1 mM MgCl2, 1 mM PMSF, and 5 µg/ml each of pepstatin A, leupeptin, aprotinin, and chymostatin. Homogenates then were sonicated and spun at 15,000 × g for 15 min. The supernatant then was spun for 1 h at 100,000 × g, and the soluble fraction was used in the immunoblot analysis.

**Real-Time Reverse Transcriptase–PCR Analysis.** To determine sGC α1 and β1 mRNA levels, the use of an α1 and β1 rat isoform-specific fluorescent detected, real-time quantitative PCR (Q-PCR) technique was used, as originally described by Gibson et al. (25). In brief, 100 ng total RNA extracted from rat tissues was used in a reverse transcriptase reaction by using Superscript reverse transcriptase (GIBCO/BRL) with a specific oligonucleotide primer corresponding to the complementary sequence of α1 or β1 sGC mRNA (sGC α1, 5′-ACACAATATGCTCGATTTGCA-3′; sGC β1, 5′-CGGGGGAAGGGTGATACGTTACA-3′). For the PCR, the negative-strand oligonucleotides remained the same, and the positive-strand oligonucleotides were: 5′-GCTTCTTATCTCGTGGACCAAA-3′ for the α1 subunit and 5′-CGGGGACCTAGTATGTCAGCGCA-3′ for the β1 subunit. A third, fluorescent-labeled, oligonucleotide probe that anneals within the amplicon for each assay also was used (5′-CAACCTGTTGACACATCCAGCTCTCCACA-3′ for the α1 assay and 5′-ACAGAGTGCTCCGCTCCAGCAG-3′ for the β1 assay). Detection of the dequenched probe was achieved by using an Applied Biosystems Prism 7700 Sequence Detector (Perkin–Elmer). The data were analyzed by using the SEQUENCE DETECTOR software. Because each assay crosses an intron/exon boundary, the possibility of chromosomal DNA artifacts in the PCR is virtually eliminated. However, tubes containing no RNA template during the reverse transcriptase reaction and tubes containing no Superscript enzyme were consistently assayed in parallel to control for DNA and buffer contamination, respectively.

Relative levels of sGC α1 and β1 mRNA were reported after normalization to 36b4 mRNA, also detected by real-time Q-PCR. Levels of 36b4 message were not significantly altered by any treatment reported here.

**Northern Blot Analysis.** Samples of poly(A)+ RNA were denatured for 10 min at 60°C in RNA loading buffer and subsequently separated on a 1% agarose denaturing gel. One microgram of poly(A)+ RNA was loaded per lane. After transferring to Duralon-UV membrane (Stratagene), membranes were prehybridized for 2 h in 50% formamide, 0.5% SDS, 6× SSC, 5× Denhardt’s solution, 0.6% dextran sulfate, and 50 µg/ml yeast tRNA solution. Membranes were first hybridized with a random primed-generated 32P-dCTP-labeled probe using a random primed DNA labeling kit (Roche Molecular Biochemicals) and a 1.3-kb cDNA from sGC α1 as a template. Blots were hybridized for 16 h at 46°C and washed twice in buffer 1 (2× SSC, 0.5% SDS) then twice in buffer 2 (1× SSC, 0.1% SDS) and exposed to x-ray film (Kodak). For sGC β1, blots were stripped and reprobed in the exact manner, using a probe generated from a 1.9-kb cDNA for sGC β1. To correct for internal variation in loading, blots also were hybridized by using a 32P-labeled cDNA to glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX).

**Immunoblot Analysis.** Total protein in the 100,000 × g supernatants was determined by using Bradford reagent (Bio-Rad). Fifty micrograms of total protein from each sample was boiled for 8 min in Laemmli sample buffer and fractioned on 10% SDS gels. Resolved proteins were transferred to poly(vinylidene difluoride) membranes (Bio-Rad) and blocked overnight in 5% nonfat dry milk. Membranes then were incubated with a mouse monoclonal anti-sGC α antibody (H6) (26, 27) and sGC α protein was detected by using the ECL detection system (Amersham Pharmacia). Protein bands were analyzed by densitometry and presented as percent of control, using the densitometric values.

**Data Analysis.** Statistical analysis of the E2 time course (Fig. 1A) was performed by ANOVA followed by the Scheffé post hoc test for comparison between groups (* indicates significant difference compared with control animals at the 0.05 level). All other statistical analyses were done by using a t test (* indicates significance from control, P < 0.01; # indicates significance from *). Where applicable, the results are presented as means ± SEM.

**Results**

**E2 Rapidly Decreases Both sGC α1 and β1 mRNA.** We first determined the influence of a physiological dose of E2 on mRNA expression levels of sGC α1 and β1 using real-time Q-PCR (see Materials and Methods). As shown in Fig. 1A, in vivo administration of E2 to ovariecotomized animals resulted in a rapid decrease of sGC α1 mRNA in the uterus. The effects were seen as early as 1 h after administration (30% decrease), and reached the lowest level at 3 h (90% decrease). Over the 24-h time course, the levels of sGC α1 mRNA began to reaccumulate, but only reached approximately 42% of control values.

Similar to the effects on sGC α1 mRNA levels, uterine sGC β1 mRNA levels also were reduced by in vivo E2 administration. Within 1 h, sGC β1 levels were diminished by almost 50%, and by 3 h to 34% of control levels. Unlike sGC α1, sGC β1 levels returned back to control steady-state levels within 24 h after E2 injection (Fig. 1A).

We also determined the effect of E2 on uterine sGC α1 and β1 mRNA levels in immature nonovariectomized animals. Three hours after the in vivo administration of E2 to nonovariectomized animals, sGC α1 and β1 mRNA levels were reduced to 15% and 32% of control values, respectively (data not shown). These results indicate that sGC α1 and β1 mRNA are similarly
regulated in ovariectomized and nonovariectomized immature animals.

The reduction of sGC α1 and β1 mRNA levels in the rat uterus after 3 h of E2 in vivo treatment is further illustrated in Fig. 1B using Northern blot analysis. Transcripts of sGC α1 (5.2 kb) and β1 (4.2 kb) were detected as stated in Materials and Methods. The sGC α1 transcript is evident in control animals and virtually disappears after 3 h of E2 treatment. The dramatic reduction of sGC β1 transcript after 3 h of E2 administration also is shown.

The glyceraldehyde-3-phosphate dehydrogenase transcript was detected to control for equal loading of samples. Fig. 1C illustrates the actual quantitative levels of sGC transcripts relative to the control housekeeping gene, 36b4, in 100 ng total uterine RNA under control (untreated) and 3-h E2-treated animals using real-time Q-PCR.

**E2-Mediated sGC α1 and β1 mRNA Inhibition Is Dose-Dependent and Is Specific to Estrogen Receptor (ER) Activation.** To confirm that the E2 reduction of uterine sGC mRNA depended on ER activation, we used three methods: (i) dose response, (ii) ER antagonist, and (iii) nonestrogenic steroid hormones. First, we determined the dose–response curve for E2 reduction of sGC mRNAs. Increasing doses of E2 were injected into ovariectomized rats, and uterine sGC α1 and β1 levels were determined after 3 h. As shown in Fig. 2, at a dose of 4 μg/kg, maximal reductions of both sGC α1 and β1 mRNA levels were observed. Some animals also were pretreated with the pure ER antagonist, ICI 182,780 (2 mg/kg), 30 min before E2 injection (40 μg/kg). Fig. 3 demonstrates that competitive inhibition of E2 binding to ER by ICI 182,780 blocks the effects of E2 on the mRNA of both sGC α1 and β1 subunits in uterine tissue. There

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was no effect of the ICI compound on the expression of these RNAs when administered alone (Fig. 3).

Finally, the effects of progesterone, 5α-dihydrotestosterone, and dexamethasone on uterine sGC mRNA levels were determined. None of the nonestrogenic steroid hormones used resulted in any significant changes in sGC mRNA levels (Fig. 4). Taken together, these results indicate that the E2-mediated effects on sGC at 3 h are specifically estrogenic and require ER activation.

Puromycin Does Not Block E2-Mediated Effects on sGC mRNA Levels. To determine whether the E2 effects on sGC mRNA levels in the uterus required the synthesis of new proteins, we used the protein synthesis inhibitor puromycin (100 mg/kg). At this dose, puromycin has been repeatedly shown to be effective at blocking new protein synthesis in both uterine and liver tissues (28–30). Puromycin was unable to block the inhibitory effect of E2 on sGC α1 and β1 mRNA levels. Furthermore, puromycin alone had no effect on sGC α1 or β1 transcripts (Fig. 5). Therefore, basal expression and E2 inhibition of sGC mRNA levels occur in the absence of any newly synthesized protein during the 3-h incubation period.

E2 Inhibits sGC α1 and β1 mRNA in Vitro. To determine whether the E2-mediated effects on sGC α1 and β1 mRNA levels occurred independent of factors released from other tissues, we treated uterine tissues in vitro with E2 (10 nM) after removing them from the intact animals. After 3 h of in vitro incubation with E2, uterine sGC α1 mRNA levels were reduced by 33% and sGC β1 by 50% when compared with tissues incubated in the absence of E2 (Fig. 6). Apparently because of nonoptimal conditions, the levels of in vitro control sGC mRNA levels were only 31% and 35% of in vivo control levels for α1 and β1, respectively (data not shown). Nevertheless, a direct effect by E2 to diminish sGC mRNA expression was observed in vitro.

E2 Decreases sGC Protein Levels. Using immunoblot analysis, we determined that E2 also results in decreased sGC protein. Fig. 7A demonstrates the effect of in vivo administration of E2 to immature ovariectomized animals on sGC α protein levels in the uterus. Overall, E2 caused uterine sGC α protein levels to diminish to approximately 30–35% of control levels between 3 and 6 h after injection. By 12–18 h after E2 administration, the sGC protein levels returned to approximately 80% of the untreated animals (Fig. 7B).

Discussion

The female reproductive tissues respond dramatically to endocrine factors, specifically the ovarian steroid hormones. In this regard, estrogens and progesterone exhibit profound physiological effects in uterine tissue with respect to growth, vascularization, and gene expression. These steroid hormones also are associated with pathological states such as uterine cancers. However, the exact molecular mechanism(s) that regulate steroid hormone-mediated uterine physiological and pathological processes are not known.

The results presented here illustrate that E2 regulates both the α1 and β1 subunits of uterine sGC by reducing their mRNA levels within 1–3 h after in vivo injection to immature rats. Significant decreases were observed at the earliest time tested (1 h). The fact that the ER antagonist, ICI 182,780, blocks this response suggests that the inhibition is an ER-mediated effect. Furthermore, this effect only occurred after the in vivo administration of E2, and not in response to the other nonestrogenic hormones progesterone, 5α-dihydrotestosterone, and dexamethasone. In
The possibility remains that E2 also represses the transcriptional activity of the genes responsible for the expression of the sGC subunits. Recently, Stoner et al. (32) demonstrated that the vascular endothelial growth factor transcript is rapidly reduced by E2 through repression of transcription in a cell culture model. E2 has been shown to repress the transcription of other genes through ER activation and may involve coactivators/repressors or the inhibition of transcription factors (33–36). These particular studies, however, targeted the more classical steroid-action time frame over many hours or days of treatment. We have shown that the E2 effects on sGC mRNA levels are very rapid, and even at 24 h post-E2 injection the levels of sGC α1 remain lower by approximately 50%. We are unsure whether this trend is caused by the metabolism of E2 because only one injection of E2 was administered. It would be interesting to determine whether the maintained reduction of sGC α1 mRNA levels after 24 h (50%) is a result of a direct mechanism similar to the classical genomic pathway stated above. In this regard, sGC α1 and β1 may be regulated independently, as we also suggested previously, despite their chromosomal proximity (22). Because the levels of sGC mRNA diminish so rapidly in response to E2 in rat uterus, it is likely that certain preexisting factors (e.g., ribonucleases or transcription factors) are responsible for the effects we have reported. Alternatively, the results also may reflect differences in the rate of turnover of sGC messages.

Recent and past evidence also has suggested that various responses to E2 may be because of a nonclassical, cell-surface E2 receptor, particularly rapid responses such as elevated cGMP levels and ion channel regulation of cell permeability. Some reports describe the activation of the mitogen-activated protein kinase (MAPK) signaling pathway and cGMP accumulation within minutes of stimulation by a membrane-impermeable form of estrogen in human vascular endothelial cells (37–41). This also could help explain the rapid effects by E2 in rat uterus on sGC mRNA. Notably, nerve growth factor-mediated decreases of sGC mRNA in PC-12 cells depend on the activation of the upstream activator of the MAPK pathway, Ras (42).

In the immature rat uterus, it is understood that the majority of rapid E2 responses on gene expression occur through the stimulation of the ERα. The recently discovered beta receptor (ERβ) is present at very low levels in the uterus, and it is not clear whether functional levels of ERβ protein are present (43–45). Zhong and coworkers (41) also have demonstrated that non-genomic effects of E2 occur through ERα stimulation. Therefore, we speculate that the effects on sGC mRNA levels are likely caused by direct stimulation of ERα in the rat uterus. We have not ruled out the possibility of a classical endocrine or paracrine effect initiated by E2 that could lead to the release of a small peptide or polypeptide growth factor that may activate a cell signaling cascade in the uterine tissue, resulting in sGC mRNA reduction. However, our results demonstrate that E2 leads to the reduction of sGC α1 and β1 mRNA in vitro, indicating that this response does not depend on the secondary release of other hormones or factors from other tissues.

It is important to note that α1 and β2 isoforms of sGC also exist. In fact, the sGC α2 subunit mRNA has been shown to be abundant in the uterus (16). Our study specifically examined the regulation of sGC α1 and β1.
We have demonstrated here that ER activation leads to the rapid regulation of both sGC α1 and β1 mRNA and sGC protein in the rat uterus after in vivo E2 administration to an animal model, and we believe it plays an important role in E2-mediated uterine physiology. The long-term goal of our study is to understand how steroid hormones exhibit their effects on sGC mRNA at the molecular level, which will allow us to discover targets for the modulation and prevention/treatment of various physiological and pathological processes and diseases.


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