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Nitric oxide sensitive-guanylyl cyclase subunit expression changes during estrous cycle in anterior pituitary glands

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Cabilla JP, Ronchetti SA, Nudler SI, Miler EA, Quinteros FA, Duvilanski BH. Nitric oxide sensitive-guanylyl cyclase subunit expression changes during estrous cycle in anterior pituitary glands. Am J Physiol Endocrinol Metab 296: E731–E737, 2009. First published January 13, 2009; doi:10.1152/ajpendo.90795.2008.—17β-Estradiol (E2) exerts inhibitory actions on the nitric oxide pathway in rat adult pituitary glands. Previously, we reported that in vivo E2 acute treatment had opposite effects on soluble guanylyl cyclase (sGC) subunits, increasing α1- and decreasing β1-subunit protein and mRNA expression and decreasing sGC activity in immature rats. Here we studied the E2 effect on sGC protein and mRNA expression in anterior pituitary gland from adult female rats to address whether the maturation of the hypothalamus-pituitary axis influences its effects and to corroborate whether these effects occur in physiological conditions such as during estrous cycle. E2 administration causes the same effect on sGC as seen in immature rats, and these effects are estrogen receptor dependent. These results suggest that E2 is the main effector of these changes. Since the sGC α-subunit increases while the sGC activity decreases, we studied if other less active isoforms of the sGC α-subunit are expressed. Here we show for the first time that sGCα2 and sGCα2β2 inhibitory (α2β2) isoforms are expressed in this gland, but only sGCα2α2 mRNA increased after E2 acute treatment. Finally, to test whether E2 effects take place under a physiological condition, sGC subunit expression was monitored over estrous cycle. sGCα1, β1, and -α2, fluctuate along estrous cycle, and these changes are directly related with E2 level fluctuations rather than to NO level variations. These findings show that E2 physiologically regulates sGC expression and highlight a novel mechanism by which E2 downregulates sGC activity in rat anterior pituitary gland.

The main estrogenic hormone 17β-estradiol (E2) plays important regulatory roles in a broad variety of biological processes, acting mainly on reproductive tissues, bone, liver, pituitary, and brain (9, 25).

Nitric oxide (NO) is a signaling molecule that freely diffuses across cellular membranes where it binds to its main intracellular receptor, soluble guanylyl cyclase (sGC). This enzyme catalyzes the formation of cGMP from GTP. Subsequently, targets of cGMP such as cGMP-dependent protein kinases, cyclic nucleotide phosphodiesterases, and cyclic nucleotide-sensitive ion channels are activated to continue the signal transduction (15, 18).

sGC is an heterodimeric enzyme and is comprised of two subunits, α and β, of which four types exist (α1, α2, β1, and β2). Both α-isofoms form a functional enzyme with the β1-subunit, although the α1β1 is the most abundant and widely expressed heterodimer, showing the greater activity (12, 13). The α2 is expressed in a more restricted pattern: in human tissues, it is present mainly in spleen, placenta, brain, and uterus; in rat, it was found in fetal brain (3). Furthermore, an inhibitory α2-β1-subunit (α2β1) and many splice variants of both isoforms of variable activity have also been identified (1, 22).

Reports indicate that E2 regulates the NO/sGC/cGMP pathway and the levels of NO and cGMP in many tissues. Several studies (21, 24) sustain an inhibitory role of E2 on the NO pathway in pituitary gland. Pituitary glands from ovariectomized rats show increased NO synthase activity and mRNA and protein levels, whereas E2 treatment reverts this condition. These E2 effects were only observed after in vivo treatment, and it has been suggested that they are indirect. In addition, it has been reported that E2 affects sGC expression and activity in uterus, PC12 cells, and hypothalamus (8, 10, 14). Previous studies from our laboratory (4) show that acute E2 treatment exerts an inhibitory effect on sGC by downregulating the sGCβ1 subunit and sGC activity but increases sGCα1 expression in anterior pituitary gland from immature rats. E2 effects on anterior pituitary sGC were observed such after in vivo as in vitro treatment, suggesting a direct effect of E2 on sGC regulation and a differential and independent regulation of both subunits. Previous evidence (4, 7) further sustains that under certain conditions α1 and β1 can be independently regulated.

Taking into account preceding results, the aim of this work was to investigate whether E2 affects sGC subunit expression and activity on pituitary gland from adult rats and to corroborate whether these effects occur in physiological conditions such as during estrous cycle. Besides, since the sGC α-subunit increases while sGC activity decreases, we studied the expression of other less active isoforms of the sGC α-subunit in the anterior pituitary gland and whether they are regulated by E2. To this end, we studied the in vivo effect of exogenous or endogenous E2 on sGC expression.

MATERIALS AND METHODS

Materials

The 7α,17β-[(9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]strad-1,3,5(10)-triene-3,17-diol (ICI 182,780) was purchased from Tocris (Bristol, UK). Z-1-[2(2-aminoethyl)-N-(2-ammonioethyl)amino]diazan-1-ium-1,2-diolate (DETRANONOate) was purchased from Cayman Chemical (Ann Arbor, MI). Leupeptin, pepsta-

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tin, PMSF, DTT, and diaminobenzidine were obtained from Alexis-US Biological (Swampscott, MA). Bradford reagent was purchased from Bio-Rad (Hercules, CA). Propylene glycol and hydrogen peroxide were from Cicarelli (Buenos Aires, Argentina). GoTaq DNA polymerase was provided by Promega (Madison, WI). TRIZol and molecular biology reagents were from Invitrogen (Carlsbad, CA). Media and reagents for cell culture were purchased from GIBCO (Rockville, MD), except for the FBS that was obtained from GBO (Buenos Aires, Argentina). Otherwise indicated, all other reagents and antibodies were obtained from Sigma-Aldrich (Buenos Aires, Argentina).

**Animals and treatments**

Adult female Wistar rats (180–200 g) were used. Animals were kept with controlled conditions of light (12:12-h light-dark cycle) and temperature (21–24°C). Food and water were supplied ad libitum. All procedures were in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

For E2 acute effects experiments, rats were injected subcutaneously in the periscapular region with 40 μg/kg body wt E2 or with vehicle alone (propylene glycol) and killed over a time course. When required, animals were injected intraperitoneally with 2 mg/kg ICI 182,780 30 min before 40 g/kg E2 administration.

Intact rats were monitored by daily (0800-0900) vaginal smears over three consecutive cycles. Animals at random stages of estrous cycle or at proestrus, estrus, or diestrus were killed by decapitation at 1700.

**Ovariectomy**

Rats were ovariectomized under ketamine (75 mg/kg; Holliday-Scott, Buenos Aires, Argentina) and xylazine (10 mg/kg; König, Buenos Aires, Argentina) anesthesia 14 days before the experiments. Sham-operated rats were used as controls.

**Cell culture**

Primary cell culture was prepared from anterior pituitary glands from ovariecetomized or sham rats killed at 14 days postsurgery. Anterior pituitary glands from each condition were pooled for each cell culture. Cells were obtained by enzymatic (trypsin/DNAse) and mechanical dispersion (extrusion through a Pasteur pipette). Cell viability was assessed by the trypan blue exclusion method. Dispersed cells were seeded onto tissue culture plates and stabilized for 48 h (37°C, 5% CO2 in air) in phenol red-free DMEM supplemented with 10% charcoal stripped FBS, 10 mM glutamine, 5.6 g/ml amphotericin B, and 25 μg/ml gentamicin.

**Cell treatment**

After the stabilization period, the medium was changed for fresh medium and cells were incubated during 6 h (37°C, 5% CO2 in air) with or without 0.5 mM DETANONate. After treatment, RNA isolation from each condition was carried out.

**Immunoblot analysis**

Anterior pituitary glands were removed and sonicated in lysis buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 μM leupeptin, 350 μM pepstatin, 0.5 mM PMSF, and 0.2 mM DTT. Sonicates were centrifuged for 20 min at 10,000 g, and the soluble fraction was used in the immunoblot analysis. Protein content from the supernatants was measured by Bradford reagent, using BSA as a standard. Twenty to thirty micrograms of total protein from each sample were boiled for 5 min in Laemmli sample buffer and were fractioned on 10% SDS-PAGE. Resolved proteins were transferred to polyvinylidene difluoride membranes and blocked for 2 d at 4°C in blocking buffer (TBS-0.05% Tween 20 and 6% nonfat dry milk). Then, membranes were coincubated overnight at 4°C with rabbit antisera anti-GCα1 (1:1,750) or β1 (1:700) subunits and anti-actin (1:1,000) in blocking buffer. Then blots were washed and incubated for 1 h at room temperature with horseradish-peroxidase conjugated goat anti-rabbit IgG (1:2,000), followed by detection of immunoreactivity with diaminobenzidine solution containing 0.01% hydrogen peroxide.

**RT-PCR and semiquantitative PCR**

**RNA isolation.** Tissues were removed and immediately homogenized with TRIZol reagent. After isolation, total RNA from tissues was spectrophotometrically quantified at 260 nm. RNA integrity was checked in formaldehyde/formamide gel electrophoresis.

**RT and PCR reactions.** First-strand cDNA was synthesized with Moloney murine leukemia virus RT in RT buffer containing 5.5 mM MgCl2, 0.5 mM dNTP, 2.5 μM random hexamers, and 3.125 U/μl Moloney murine leukemia virus RT. Reactions were done in a final volume of 12 μl containing 1 μg RNA. The RT reaction was run at 37°C for 50 min, and RT was inactivated by heating the samples at 70°C for 15 min before the PCR reactions. To check for genomic contamination, the same procedure was performed on a reaction containing no RT.

Specific primers for both subunits of sGC were designed from published sequences (23) with Oligo Perfect designer software (Invitrogen) and are detailed in Table 1. The amplified products spanned from nucleotide position base 1,971 to 2,054 in the C-terminal region of sGCα1, from 714–823 in the N-terminal region of sGCβ1, and from within the in-frame insert to 275 bp to the 3’-end of sGCβ2 (1). β-Actin was used as an endogenous control. Actin primers were designed to detect amplification of DNA contamination. Then, samples were thermocycled for PCR amplification (Mastercycler; Eppendorf, Hamburg, Germany). The reaction mixture contained GoTaq PCR buffer, 1.5 mM MgCl2, 200 μM of each dNTP, 0.625 U GoTaq polymerase, and 300 nM of each primer. RT-PCR methods were utilized to determine relative changes in mRNA expression. Reactions were subjected to a varying number (n = 16–40) of cycles of PCR amplification (melting phase 94°C for 30 s, annealing 55°C for 30 s, and extension 72°C for 1 min) to find out the optimum cycle number within the linear range for PCR amplification. Amplified products collected at various cycles were analyzed by electrophoresis in 1.5% agarose-ethidium bromide gels, and the optimum cycle number resulted in 24 cycles for sGCα1 and sGCβ1, 399 bp for sGCβ2, and 275 bp for sGCα2 and sGCβ2.

**Analysis of semiquantitative PCR and immunoblot data**

The intensities of PCR products and immunoblot signals were determined by digital image analysis using the Gel Pro analyzer

Table 1. Primers used for semiquantitative RT-PCR assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>sGCα1</td>
<td>399 bp</td>
</tr>
<tr>
<td>Forward</td>
<td>GCTCTCTATATGACAAAAGATG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGAGGTCTTTACGGATGTCAACG-5’</td>
</tr>
<tr>
<td>sGCβ1</td>
<td>399 bp</td>
</tr>
<tr>
<td>Forward</td>
<td>GCGACTGTCTACCCCGTTTGTGAT-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>ACCACAGCTGAGAGGGAAATCG-5’</td>
</tr>
<tr>
<td>sGCβ2</td>
<td>275 bp</td>
</tr>
<tr>
<td>Forward</td>
<td>GCGACGCGCGGAATGAATG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>ACGAGACCGCGGAATGAATG-5’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>276 bp</td>
</tr>
<tr>
<td>Forward</td>
<td>ACCCAAGCTGAGAAAGGACTG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGAGGCCTTGTAAATGCAAGC-5’</td>
</tr>
</tbody>
</table>
Differential E₂ Effects on sGC Subunits Expression

E₂ Administration Increased sGCα₁ and Decreased sGCβ₁ Protein and mRNA Expression in Anterior Pituitary Glands of Adult Female Rats, Decreasing cGMP Production

To Identify Whether the Maturation of the Hypothalamus-pituitary axis influences E₂ effects on sGC subunits, we studied the influence of a physiological dose of E₂ (4, 14) on sGC subunit expression in adult female rats. Rats at random estral stages (n = 5 per group) received a single dose of 10⁻¹⁴ M E₂ and were killed over a time course. As seen previously in immature rats (4), in vivo administration of E₂ resulted in an increase of sGCα₁ levels and, concomitantly, a decrease in sGCβ₁ protein levels. These effects became evident as soon as 4 h after E₂ administration and were maximal at 8 h (relative units as percentage of control, 8 h: E₂; sGCα₁: 143 ± 11 and sGCβ₁: 79 ± 8; P < 0.05, ANOVA followed by Dunnett’s test). Protein levels of both subunits tended to return to control values after 12–16 h post-E₂ injection (data not shown). Since these changes in protein expression could respond to altered mRNA synthesis, we evaluated then the action of E₂ on sGCα₁ and sGCβ₁ mRNA expression by semiquantitative PCR. At 4- and 8-h post-E₂ injection, sGCα₁ mRNA was significantly augmented respect to control values (Fig. 1). At the same time points, sGCβ₁ mRNA levels were diminished, consistent with the observed at protein level. These findings indicate that the differences observed at the protein level are consequence of effects at the transcription level.

Given that both subunits are required at 1:1 stoichiometry to have cGMP-producing activity, cGMP production was measured at different times. sGC activity was significantly reduced after 8 h of treatment with E₂ [concentration of GMP (fmol/mg prot): means ± SE; control: 160 ± 23; E₂ 8 h: 220 ± 38, and E₂ 8 h: 98 ± 21; P < 0.05 vs. control, ANOVA followed by Dunnett’s test; n = 3], when the difference between subunit expression became maximal (data not shown). These results suggest that, independently of sGCα₁ subunit increase, E₂-mediated downregulation of β₁ would be enough to decrease cGMP in the pituitary.

E₂ effects are estrogen receptor dependent

Next, to test if the actions of E₂ on sGC subunits levels were specific, rats were injected with the pure estrogen receptor (ER) antagonist ICI 182,780 (2 mg/kg ip) 30 min before E₂ administration and killed after 8 h. The antagonist had no effect by itself, but when coadministered with E₂, it was able to completely avoid E₂ effects on both sGC subunit protein levels (Fig. 2). This observation indicates that the E₂ effects on sGC expression are mediated by ER activation.

It is known that other isoforms of sGCα are expressed in different tissues. To date, the presence of these isoforms was not reported in pituitary gland. In our case, the augment registered in sGCα₁ protein as well as in mRNA could be due to an increase in α₁ and/or to other less active or inhibitory isoforms of sGCα. In the present work, the anti-sGCα₁ antibody utilized for immunoblot studies, as well as the primers used to amplify sGCα₁ mRNA, was directed to the C-terminal sequence of rat sGCα₁ and it cannot differentiate among sGCα species, since all of them include this sequence. To examine if these subunits are expressed in this tissue, we performed RT-PCR to detect sGCα₂ and α₂₂, using specific primers and liver and kidney or spleen as control tissues, respectively. Here we show for the first time that sGCα₂ and α₂₂ are expressed in anterior pituitary gland (Fig. 3A). Then, to investigate if their expression was modified by E₂ treatment, rats were treated with a single dose of E₂ and killed after 6 h. E₂ treatment did not modify α₂ mRNA expression respect to control, but α₂₂ mRNA levels were dramatically increased (relative units as percentage of control; sGCα₂ control: 100 ± 24; E₂ 8 h: 94 ± 30; sGCα₂ control: 100 ± 18; and E₂ 8 h: 1,730 ± 105; P < 0.001, Dunnett’s test; n = 3; Fig. 3B). Again, to verify if this effect was E₂ specific, rats were injected intraperitoneally with 2 mg/kg ICI 182,780 30 min before E₂ subcutaneous administration, and killed over 8 h. The inhibitor was able to fully 

Figure 1. Acute E₂ treatment increases α₁ soluble guanylyl cyclase (sGC) but decreases β₁ sGC mRNA expression in rat anterior pituitary gland. Adult intact rats at random stages of estrous cycle (n = 5 per group) were injected subcutaneously with 40 μg/kg body wt E₂ and killed after 8 h. Top: representative semiquantitative PCR. Bottom: average densitometric values. Bars are means ± SE of the densitometric values of sGCα₁ (open bars) and sGCβ₁ (solid bars) mRNA densitometric values normalized to actin, as percentage of control (n = 5). *P < 0.05, **P < 0.01 vs. control, ANOVA followed by Dunnett’s test.
abolish α2i expression increase and had no effect per se on sGCα2i (Fig. 3C). Thus the augmented sGCα expression may reflect α1-increased levels but also an augmented α2i expression. These findings suggest that both β1 downregulation and α2i-augmented expression could contribute to the acute inhibitory effect of E2 on sGC.

sGC subunit expression levels are variable through estrous cycle

Taking into account the acute E2 actions on sGC expression and activity and to examine whether these effects occur under physiological conditions, we studied the changes on sGC through estrous cycle, a condition where E2 and also gonado-trophins, prolactin, and other gonadal steroids undergo rapid, dramatic changes. To this end, rats were killed on the afternoon (1700) of each stage of the estrous cycle and sGC protein levels were analyzed by Western blot. Protein levels of sGCα1 showed a significant increase through the cycle from proestrus to diestrus (Fig. 4A). sGCβ1 protein levels, which were higher than sGCα1 on the afternoon of proestrus, did not change during estrus but significantly decreased at diestrus. Similar results were observed when sGCα1 and β1 mRNA expression was evaluated by RT-PCR (data not shown). These findings show that sGC subunits levels independently fluctuate in vivo during estrous cycle. These individual variations of sGC subunit expression are consequence of hormonal changes taking place during estrous cycle, because in male rats and in ovariectomized rats both subunits show similar mRNA levels (relative units expressed as means ± SE; n = 3; male sGCα1: 1.41 ± 0.15 and sGCβ1: 1.54 ± 0.2; and 14-day ovariectomized sGCα1: 1.43 ± 0.42 and sGCβ1: 1.61 ± 0.23).

sGCα2i expression levels are variable through the estrous cycle

Bearing in mind our findings showing that sGCα1 expression increases over the cycle in spite of the decrease in cGMP production, the sGCα2i contribution to global expression was addressed. In accordance with the enhanced sGC activity on proestrus, α2i mRNA expression was the lowest at this stage (Fig. 4B). On estrus and diestrus, α2i expression was significantly augmented, which correlates with a lesser cGMP production. Altogether, the difference between sGC subunit expression according to the stage of the estrous cycle in which the animals were killed suggests the cyclicity of the response.

Role of NO on sGC subunit expression

NOS I protein and activity are strikingly upregulated on the afternoon of proestrus, and both return to basal levels after the afternoon of estrous, remaining low on diestrus I and II. cGMP production strongly correlates with NOS I upregulation (17). To determine if the changes seen in sGC subunit protein levels were due to changes in NO levels, we studied the in vitro effect of a short-time NO and E2 exposition on sGC protein expression. Pituitary cell cultures from intact adult female rats were incubated with 0.1 mM DETANONOHate, a NO donor, or 10⁻⁹ M E2 for 6 h, and sGCα1 and β1 expression was evaluated by Western blot. NO treatment upregulated both subunits in a
similar fashion (Fig. 5A) while E2 treatment increased \( \alpha_1 \) but decreased \( \beta_1 \) expression (Fig. 5B). In addition, sGC\( \alpha_1 \) and \( \beta_1 \) expression was studied in the anterior pituitary glands of 14-day ovariectomized rats when NOS I protein and activity were markedly upregulated. At this condition, the results were similar to those with the NO donor in vitro (relative units as percentage of control; sGC\( \alpha_1 \): 120.7 ± 7.5 and sGC\( \beta_1 \): 126 ± 10.7; \( P < 0.05 \), ANOVA followed by Dunnett’s test; \( n = 3 \)). Thus short-time in vitro NO treatment or chronic in vivo NO increase did not mimic the effect of E2 in vitro or those seen in vivo during estrous cycle on sGC subunits. These results suggest again that the imbalance of sGC subunit expression is likely due to hormonal fluctuations rather than to NO.

**DISCUSSION**

Here we have shown that E2 causes the same effect on sGC subunits in adult and juvenile female animals, suggesting that these effects are distinctive of E2 and independent of the hormonal fluctuation during the maturation of the reproductive axis. In vivo E2 acute treatment exerted opposite actions on both sGC subunits, increasing sGC\( \alpha_1 \) mRNA and protein levels and, simultaneously, decreasing sGC\( \beta_1 \) mRNA and protein levels. These effects were E2 specific and ER dependent. Our results also demonstrate that \( \alpha_1 \)- and \( \beta_1 \)-subunit levels are independently modified. Different authors have previously reported that under certain conditions \( \alpha_1 \) and \( \beta_1 \)-subunits can be individually regulated. A complete loss of the \( \beta_1 \)-subunit in aortic smooth muscle cells from old rats has been found, while the \( \alpha_1 \)-subunit is still present (7). In developing rat brain, only sGC\( \alpha \) mRNA is expressed, while sGC\( \beta_1 \) is totally absent (26), raising interesting questions regarding the independent functions of each subunit.

We have demonstrated that E2 acute treatment enhanced \( \alpha_1 \) mRNA and protein expression but decreased sGC activity. It is known that fully active sGC requires \( \alpha_1 \)- and \( \beta_1 \)-subunits in a strict 1:1 stoichiometry, and, even considering that \( \alpha_1 \) expression was increased, sGC\( \beta_1 \) downregulation would be enough to reduce sGC global activity. However, the higher expression of sGC could be due, at least in part, to an increased expression of other \( \alpha \)-isoforms with less activity. In the present work, we have demonstrated for the first time that sGC\( \alpha_2 \) and sGC\( \alpha_3 \) isoforms are expressed in anterior pituitary gland and that after E2 stimulus sGC\( \alpha_2 \) expression (but not \( \alpha_2 \)) is augmented. The
fact that the inhibitory α isoform could collaborate to E2 transient sGC inhibition indicates again that E2 is acting through multiple pathways. Therefore, our results show that E2 not only decreases sGCβ1 expression but also stimulates sGCα2 expression and by these ways participates in sGC activity downregulation.

The estrous cycle is a physiological event in which mainly E2 levels, among other hormones, suffer strong changes (27). We studied the expression of both sGC subunits during the estrous cycle to address whether the changes in E2 levels are reflected in the expression pattern of sGC in anterior pituitary gland. Results from this work show that sGCα- and β-subunits levels fluctuate through estrous cycle, further supporting a correlation between E2 level changes and sGC expression pattern. On diestrus, when E2 levels are rising and NO production is at baseline levels, the expression of sGC/H9251 is detected (6, 19, 28). Thus the putative role of proestrus, when the highest levels of apoptotic cells are detected (15-17, 32), is that the antidepressant-like effect of estradiol is due to the increase in NO production. The study of the roles of each subunit of sGC in other processes, in addition to the classical cGMP-producing function, will raise new perspectives and novel pathways to a well-known enzyme.

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