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

Jimena P. Cabilla, Sonia A. Ronchetti, Silvana I. Nudler, Eliana A. Miler, Fernanda A. Quinteros, and Beatriz H. Duvilanski

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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.—17 β -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 α_1 - and decreasing β_1 -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 sGC α_2 and sGC α_{2i} inhibitory (α_{2i}) isoforms are expressed in this gland, but only sGC α_{2i} 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. sGC α_1 , - β_1 , and - α_{2i} 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.

estrogen; soluble guanylyl cyclase; inhibitory subunit

THE MAIN ESTROGENIC HORMONE 17 β -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 (α_1 , α_2 , β_1 , and

β_2). Both α -isoforms form a functional enzyme with the β_1 -subunit, although the $\alpha_1\beta_1$ is the most abundant and widely expressed heterodimer, showing the greater activity (12, 13). The α_2 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 α_2 -subunit (α_{2i}) and many splicing 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 sGC β_1 subunit and sGC activity but increases sGC α_1 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 α_1 and β_1 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 7 α ,17 β -{9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl}stra-1,3,5(10)-triene-3,17-diol (ICI 182,780) was purchased from Tocris Neuramin (Bristol, UK). Z-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETANONOate) 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 µg/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 ovariectomized 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 µl/ml MEM amino acids, 2 mM glutamine, 5.6 µg/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 DETANONOate. 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 samples in a reaction solution lacking 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, from 1,530–1,929 in the C-terminal region of sGCα₂, and from within the in-frame insert to 275 bp to the 3'-end of sGCα_{2i} (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 β-actin, 28 cycles for sGCα₁ and β₁, and 40 cycles for sGCα₂ and sGCα_{2i}.

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

Gene	Primer	Product Size
sGCα ₁	Forward 5'-ACACAATATGCATCTCCGATGG-3'	83 bp
	Reverse 5'-GCTCTCTATACTCGCTTTGACCAA-3'	
sGCβ ₁	Forward 5'-CCCCTGGAAACTGATGTCAA-3'	109 bp
	Reverse 5'-CGGGACCTAGTAGTCACGCA-3'	
sGCα ₂	Forward 5'-GCGACTGTCTACCCGGTTTGTGAT-3'	399 bp
	Reverse 5'-CTGTACTTGTCTGCCCTTGCCATAA-3'	
sGCα _{2i}	Forward 5'-TTTTCTCCTTTCTGTTTCCATCC-3'	275 bp
	Reverse 5'-ACGAGACCGGGAATGAATG-3'	
β-Actin	Forward 5'-ACCACAGCTGAGAGGGAAATCG-3'	276 bp
	Reverse 5'-AGAGGTCTTTACGGATGTCAACG-3'	

sGC, soluble guanylyl cyclase; i, inhibitory isoform.

(Media Cybernetics, Silver Spring, MD) software for Windows. To allow statistical comparison of results from different experiments, sGC subunits levels were normalized to the value of the β -actin amplified band in each lane or to the actin immunoreactive band, respectively.

Intracellular cGMP determination

Anterior pituitary glands were quickly removed and placed on dry ice. Subsequently, they were sonicated in warm 50 mM sodium acetate pH 6.2, boiled for 10 min, and centrifuged at 10,000 *g* for 10 min. Supernatants were stored at -70°C until cGMP determination. Cyclic GMP was assayed as previously described (2) by specific RIA using rabbit anti-cGMP polyclonal antiserum and acetylated cGMP as standard. Total protein content in the pellets was measured as described above.

Statistical analysis

Results are expressed as means \pm SE and were evaluated by one-way ANOVA followed by Dunnett's, Tukey's, or Student's *t*-test, depending on the experimental design. Differences between groups were considered significant if $P < 0.05$. Results were confirmed by at least three independent experiments.

RESULTS

E₂ Administration Increased sGC α_1 and Decreased sGC β_1 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^{-9} 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 sGC α_1 levels and, concomitantly, a decrease in sGC β_1 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; sGC α_1 : 143 ± 11 and sGC β_1 : 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 sGC α_1 and sGC β_1 mRNA expression by semiquantitative PCR. At 4- and 8-h post-E₂ injection, sGC α_1 mRNA was significantly augmented respect to control values (Fig. 1). At the same time points, sGC β_1 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 \pm SE; control: 160 ± 23 , E₂ 4 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 sGC α_1 subunit increase, E₂-mediated downregulation of β_1 would be enough to decrease cGMP in the pituitary.

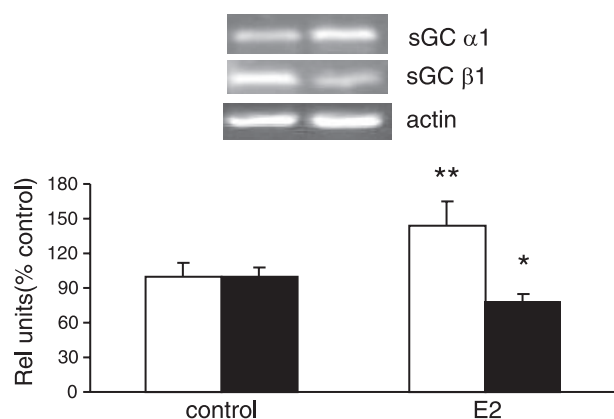


Fig. 1. Acute E₂ treatment increases α_1 soluble guanylyl cyclase (sGC) but decreases β_1 sGC mRNA expression in rat anterior pituitary gland. Adult intact rats at random stages of estrous cycle ($n = 5$ per group) were injected subcutaneous with 40 $\mu\text{g}/\text{kg}$ body wt E₂ and killed after 8 h. *Top*: representative semiquantitative PCR. *Bottom*: average densitometric values. Bars are means \pm SE of the densitometric values of sGC α_1 (open bars) and sGC β_1 (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.

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.

E₂ acute treatment increases the expression of the inhibitory subunit α_{2i}

It is known that other isoforms of sGC α 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 sGC α_1 protein as well as in mRNA could be due to an increase in α_1 and/or to other less active or inhibitory isoforms of sGC α . In the present work, the anti-sGC α_1 antibody utilized for immunoblot studies, as well as the primers used to amplify sGC α_1 mRNA, was directed to the C-terminal sequence of rat sGC α_1 and it cannot differentiate among sGC α species, since all of them include this sequence. To examine if these subunits are expressed in this tissue, we performed RT-PCR to detect sGC α_2 and α_{2i} , using specific primers and liver and kidney or spleen as control tissues, respectively. Here we show for the first time that sGC α_2 and α_{2i} 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 α_2 mRNA expression respect to control, but α_{2i} mRNA levels were dramatically increased (relative units as percentage of control; sGC α_2 control: 100 ± 24 ; E₂ 8 h: 94 ± 30 ; sGC α_{2i} control: 100 ± 18 ; and E₂ 8 h: $1,730 \pm 105$; $P < 0.001$, 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

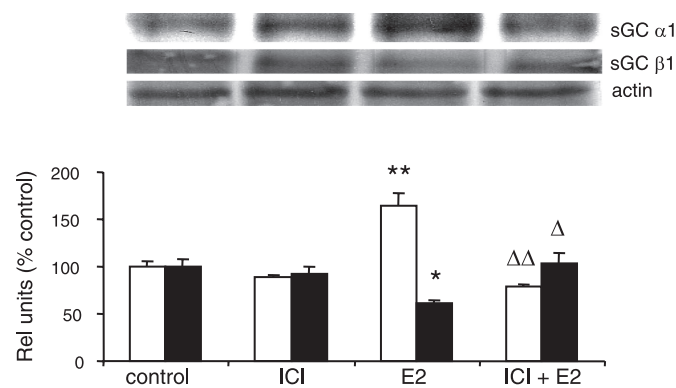


Fig. 2. E₂ actions on sGC protein expression are abolished by pure anti-estrogenic ICI 182,780 pretreatment in rat anterior pituitary gland. Adult intact rats at random stages of estrous cycle ($n = 5$ per group) were injected subcutaneous with 2 mg/kg ICI 182,780 (ICI) 30 min before 40 μ g/kg body wt E₂ injection and killed after 8 h. *Top*: representative Western blot. *Bottom*: average densitometric values. Bars represent media \pm SE of the densitometric values of sGC α_1 (open bars) and sGC β_1 (solid bars) protein levels normalized to actin, as percentage of control ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. respective controls; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ vs. E₂, ANOVA followed by Dunnett's test.

abolish α_{2i} expression increase and had no effect per se on sGC α_{2i} (Fig. 3C). Thus the augmented sGC α expression may reflect α_1 -increased levels but also an augmented α_{2i} expression. These findings suggest that both β_1 downregulation and α_{2i} -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 gonadotrophins, 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 sGC α_1 showed a significant increase through the cycle from proestrus to diestrus (Fig. 4A). sGC β_1 protein levels, which were higher than sGC α_1 on the afternoon of proestrus, did not change during estrus but significantly decreased at diestrus. Similar results were observed when sGC α_1 and β_1 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 \pm SE; $n = 3$; male sGC α_1 : 1.41 ± 0.15 and sGC β_1 : 1.54 ± 0.2 ; and 14-day ovariectomized sGC α_1 : 1.43 ± 0.42 and sGC β_1 : 1.61 ± 0.23).

sGC α_{2i} expression levels are variable through the estrous cycle

Bearing in mind our findings showing that sGC α_1 expression increases over the cycle in spite of the decrease in cGMP production, the sGC α_{2i} contribution to global expression was

addressed. In accordance with the enhanced sGC activity on proestrus, α_{2i} mRNA expression was the lowest at this stage (Fig. 4B). On estrus and diestrus, α_{2i} 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 process.

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 estrus, 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 DETANONOate, a NO donor, or 10^{-9} M E₂ for 6 h, and sGC α_1 and β_1 expression was evaluated by Western blot. NO treatment upregulated both subunits in a

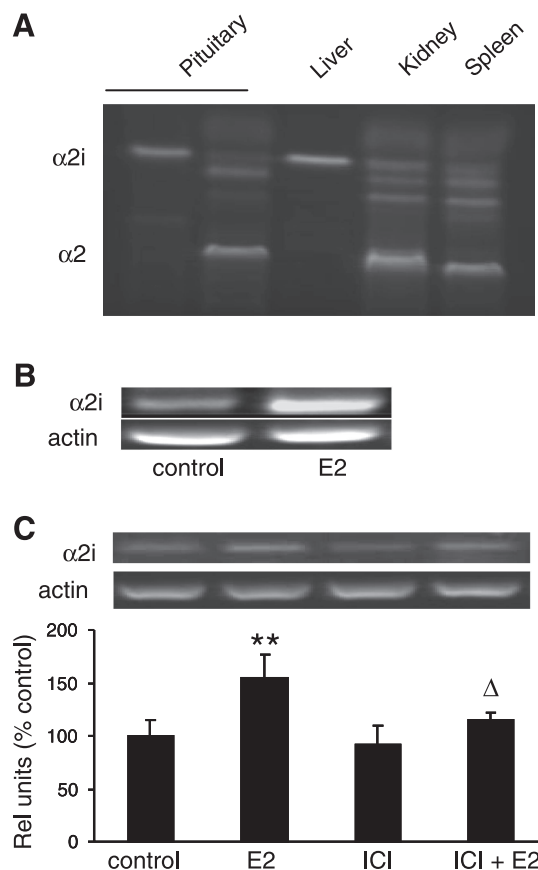


Fig. 3. Other isoforms of sGC α are expressed normally in rat anterior pituitary gland. *A*: representative PCR showing the presence of sGC α_2 and α_{2i} mRNAs in anterior pituitary. Liver, kidney, and spleen were used as respective control tissues. *B*: acute E₂ exposition increases the mRNA expression of the dominant negative isoform sGC α_{2i} . Animals were injected with 40 μ g/kg body wt E₂ and killed after 8 h. *C*: role of ER in sGC α_{2i} -augmented expression. Adult intact rats at random stages of estrous cycle ($n = 3$ per group) were injected intraperitoneally with 2 mg/kg ICI 182,780 30 min before 40 μ g/kg body wt E₂ injection and killed after 8 h. Bars are means \pm SE of the densitometric values of sGC α_i mRNA normalized to actin, as percentage of control ($n = 3$). * $P < 0.05$ vs control; $\Delta P < 0.05$ vs. E₂, ANOVA followed by Tukey's test.

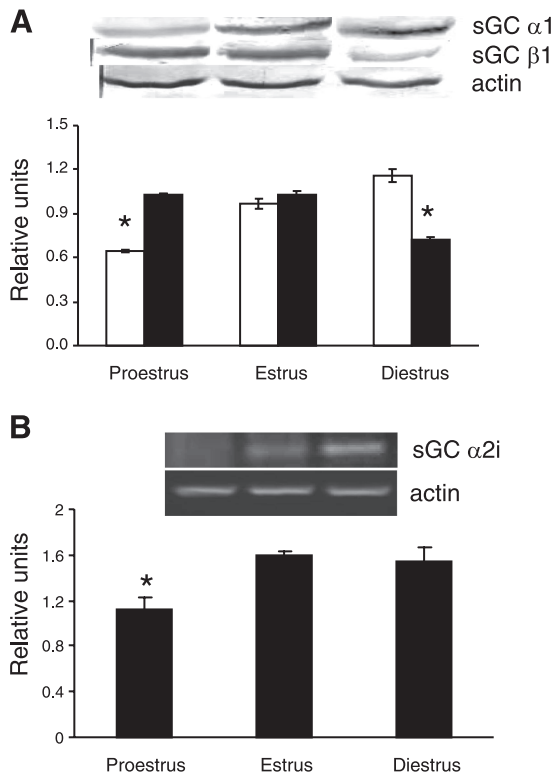


Fig. 4. sGC subunit expression fluctuates over estrous cycle. Rats ($n = 3$ per group) were killed at 1700 on proestrus, estrus, or diestrus. *A*, top: a representative Western blot; bottom: average densitometric values. Bars are means \pm SE of sGC α_1 (open bars) and sGC β_1 (solid bars) protein values normalized to actin ($n = 3$). * $P < 0.05$ vs. estrus, ANOVA followed by Tukey's test. *B*, top: a representative PCR of sGC α_{2i} ; bottom: average densitometric values. Bars are means \pm SE of sGC α_{2i} mRNA levels normalized to actin ($n = 3$). * $P < 0.05$ vs. estrus and diestrus, ANOVA followed by Tukey's test.

similar fashion (Fig. 5A) while E₂ treatment increased α_1 but decreased β_1 expression (Fig. 5B). In addition, sGC α_1 and β_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 α_1 : 120.7 ± 7.5 and sGC β_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 α_1 mRNA and protein levels and, simultaneously, decreasing sGC β_1 mRNA and protein levels. These effects were E₂ specific and ER dependent.

Our results also demonstrate that α - and β -subunit levels are independently modified. Different authors have previously reported that under certain conditions α - and β -subunits can be individually regulated. A complete loss of the β_1 -subunit in aortic smooth muscle cells from old rats has been found, while the α -subunit is still present (7). In developing rat brain, only sGC α mRNA is expressed, while sGC β_1 is totally absent (26), raising interesting questions regarding the independent functions of each subunit.

We have demonstrated that E₂ acute treatment enhanced α_1 mRNA and protein expression but decreased sGC activity. It is known that fully active sGC requires α - and β -subunits in a strict 1:1 stoichiometry, and, even considering that α_1 expression was increased, sGC β_1 downregulation would be enough to reduce sGC global activity. However, the higher expression of α GC could be due, at least in part, to an increased expression of other α -isoforms with less activity. In the present work, we have demonstrated for the first time that sGC α_2 and sGC α_{2i} isoforms are expressed in anterior pituitary gland and that after E₂ stimulus sGC α_{2i} expression (but not α_2) is augmented. The

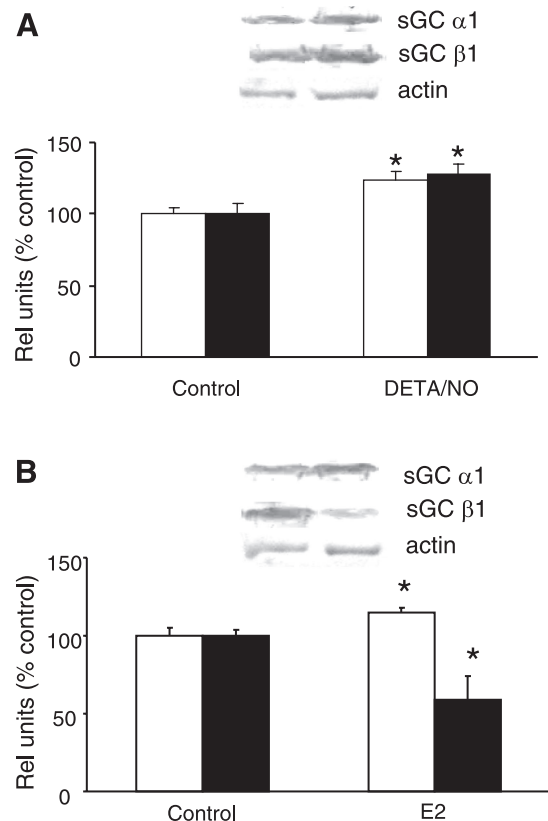


Fig. 5. Short-time nitric oxide exposure upregulated sGC expression, while E₂ acute expression differentially affects sGC subunits in vitro. *A*: primary pituitary cell cultures from adult intact rats at random stages of estrous cycle were incubated with 0.5 mM DETANONOate, a nitric oxide donor, or with culture medium alone (control) for 6 h. Top: representative Western blot. Bottom: average densitometric values. Bars are media \pm SE of sGC α_1 (open bars) or sGC β_1 (solid bars) protein levels normalized to actin, as percentage of control ($n = 5$). *B*: primary pituitary cell cultures from intact, adult rats were incubated with 10^{-9} M E₂ or with culture medium alone (control) for 6 h. Top: representative Western blot. Bottom: average densitometric values. Bars are means \pm SE of sGC α_1 (open bars) or sGC β_1 (solid bars) protein levels normalized to actin, as percentage of control ($n = 3$). * $P < 0.05$ vs. respective control, Student's *t*-test.

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 sGC β_1 expression but also stimulates sGC α_{2i} 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 sGC α - 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 β_1 is the lowest of the entire cycle and, conversely, sGC α_1 expression is maximal. Later, on the afternoon of proestrus, when serum E₂ levels have drastically fallen and NO production is high, sGC α_1 expression decreases, while a marked increase in sGC β_1 expression is observed. However, the changes in sGC subunit expression are not identical to that obtained after E₂ acute administration; there is a shifting towards diestrus where the maximal expression of α_1 was observed. It is possible that the slow but continuous increase in serum E₂ levels, beginning on diestrus I, would be enough to cause the differential changes in the sGC subunits on diestrus.

Our results show that E₂ treatment enhances sGC α expression, including an augment in α_{2i} . However, the proportion of α_{2i} vs. α_1 expression is very low. Why does E₂ augment sGC α_1 expression if it would not be involved in sGC enzyme constitution? There is some evidence that strongly suggests that the sGC α_1 subunit can be individually involved in other processes, independently of cGMP production. In advanced prostate cancer, sGC α_1 levels are highly correlated with proliferation (5). E₂ as a mitogenic factor, as well as a proapoptotic stimulus, drives changes in pituitary cell population (11, 16, 20). sGC α_1 levels and timing of pituitary cell proliferation seem to be correlated. sGC α_1 expression raises on estrus and diestrus, while proliferation events are taking place, and remains low on proestrus, when the highest levels of apoptotic cells are detected (6, 19, 28). Thus the putative role of α_1 in anterior pituitary cell renewal opens a very attractive landscape that is now under investigation.

NO modifies sGC expression on various tissues (15), and NOS expression and activity in anterior pituitary are variable and susceptible of regulation during estrous cycle (17). Here we show that both short- and long-time NO exposition upregulated both sGC subunits at the same extent. Therefore, NO seems not to be involved in the differential changes of the sGC subunit expression during the estrous cycle.

In summary, we provide evidence that sGC activity is downregulated and its subunits are independently affected upon stimulus with exogenous E₂ and during the estrous cycle in the rat anterior pituitary gland. These effects appear to be independent of the hypothalamic-pituitary-gonadal axis maturation. The expression of other less active or inhibitory sGC α isoforms provides a new potential regulation point in sGC activity. The study of the roles of each subunit of sGC in other processes, in addition to the classical cGMP-producing func-

tion, will raise new perspectives and novel pathways to a well-known enzyme.

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