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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 (E_2) exerts inhibitory actions on the nitric oxide pathway in rat adult pituitary glands. Previously, we reported that in vivo E_2 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 E_2 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. E_2 administration causes the same effect on sGC as seen in immature rats, and these effects are estrogen receptor dependent. These results suggest that E_2 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 sGCα_2 and sGCα_2 inhibitory (α_{2i}) isoforms are expressed in this gland, but only sGCα_{2i} mRNA increased after E_2 acute treatment. Finally, to test whether E_2 effects take place under a physiological condition, sGC subunit expression was monitored over estrous cycle. sGCα_1, -β_1, and -α_{2i} fluctuate along estrous cycle, and these changes are directly related with E_2 level fluctuations rather than to NO level variations. These findings show that E_2 physiologically regulates sGC expression and highlight a novel mechanism by which E_2 downregulates sGC activity in rat anterior pituitary gland.

estrogen; soluble guanylyl cyclase; inhibitory subunit

THE MAIN ESTROGENIC HORMONE 17β-estradiol (E_2) 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 E_2 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 E_2 on the NO pathway in pituitary gland. Pituitary glands from ovariectomized rats show increased NO synthase activity and mRNA and protein levels, whereas E_2 treatment reverts this condition. These E_2 effects were only observed after in vivo treatment, and it has been suggested that they are indirect. In addition, it has been reported that E_2 affects sGC expression and activity in uterus, PC12 cells, and hypothalamus (8, 10, 14). Previous studies from our laboratory (4) show that acute E_2 treatment exerts an inhibitory effect on sGC by downregulating the sGCβ_1 subunit and sGC activity but increases sGCα_1 expression in anterior pituitary gland from immature rats. E_2 effects on anterior pituitary sGC were observed such after in vivo as in vitro treatment, suggesting a direct effect of E_2 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 E_2 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 E_2. To this end, we studied the in vivo effect of exogenous or endogenous E_2 on sGC expression.

MATERIALS AND METHODS

Materials

The 7α,17β-{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]diazen-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 polimerase 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 E_2 acute effects experiments, rats were injected subcutaneously in the periscapular region with 40 μg/kg body wt E_2 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 E_2 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 xylacine (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% CO_2 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% CO_2 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-GCα_1 (1:1,750) or β_1 (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 $MgCl_2$, 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 sGCα_1, from 714–823 in the N-terminal region of sGCβ_1, from 1,530–1,929 in the C-terminal region of sGCα_2, 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 $MgCl_2$, 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 sGCα_1 and β_1, and 40 cycles for sGCα_2 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′-CCCGTGGAAACTGATGTCAA-3′	109 bp
Reverse	5′-CGGGACCTAGTAGTCACGCA-3′	
sGCα_2		
Forward	5′-GCGACTGTCTACCCCGTTTGTGAT-3′	399 bp
Reverse	5′-CTGTACTTGCTGCCCTTGCCATAA-3′	
sGCα_{2i}		
Forward	5′-TTTTCTCCTTTCCTGTTTCCATCC-3′	275 bp
Reverse	5′-ACGAGACCGCGGAATGAATG-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 ± 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

E_2 Administration Increased sGCα_1 and Decreased sGCβ_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 E_2 effects on sGC subunits, we studied the influence of a physiological dose of E_2 (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 E_2 and were killed over a time course. As seen previously in immature rats (4), in vivo administration of E_2 resulted in an increase of sGCα_1 levels and, concomitantly, a decrease in sGCβ_1 protein levels. These effects became evident as soon as 4 h after E_2 administration and were maximal at 8 h (relative units as percentage of control, 8-h E_2; sGCα_1: 143 ± 11 and sGCβ_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-E_2 injection (data not shown). Since these changes in protein expression could respond to altered mRNA synthesis, we evaluated then the action of E_2 on sGCα_1 and sGCβ_1 mRNA expression by semiquantitative PCR. At 4- and 8-h post-E_2 injection, sGCα_1 mRNA was significantly augmented respect to control values (Fig. 1). At the same time points, sGCβ_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 E_2 [concentration of GMP (fmol/mg prot); means ± SE; control: 160 ± 23, E_2 4 h: 220 ± 38, and E_2 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 sGCα_1 subunit increase, E_2-mediated downregulation of β_1 would be enough to decrease cGMP in the pituitary.

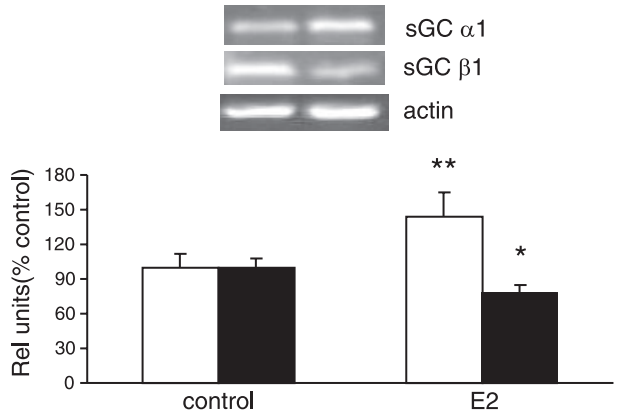

Fig. 1. Acute E_2 treatment increases α_1 soluble guanylyl cyclase (sGC) but decreases β_1 sGC mRNA expression in rat anterior pituitary gland. Adult intact rats at random stages of estrous cycle ($n = 5$ per group) were injected subcutaneous with 40 μg/kg body wt E_2 and killed after 8 h. *Top*: representative semiquantitative PCR. *Bottom*: average densitometric values. Bars are means ± SE of the densitometric values of sGCα_1 (open bars) and sGCβ_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.

E_2 effects are estrogen receptor dependent

Next, to test if the actions of E_2 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 E_2 administration and killed after 8 h. The antagonist had no effect by itself, but when coadministered with E_2, it was able to completely avoid E_2 effects on both sGC subunit protein levels (Fig. 2). This observation indicates that the E_2 effects on sGC expression are mediated by ER activation.

E_2 acute treatment increases the expression of the inhibitory subunit α_{2i}

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 sGCα_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 sGCα. In the present work, the anti-sGCα_1 antibody utilized for immunoblot studies, as well as the primers used to amplify sGCα_1 mRNA, was directed to the C-terminal sequence of rat sGCα_1 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 sGCα_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 sGCα_2 and α_{2i} are expressed in anterior pituitary gland (Fig. 3*A*). Then, to investigate if their expression was modified by E_2 treatment, rats were treated with a single dose of E_2 and killed after 6 h. E_2 treatment did not modify α2 mRNA expression respect to control, but α_{2i} mRNA levels were dramatically increased (relative units as percentage of control; sGCα_2 control: 100 ± 24; E_2 8 h: 94 ± 30; sGCα_{2i} control: 100 ± 18; and E_2 8 h: 1,730 ± 105; $P < 0.001$, Dunnett's test; $n = 3$; Fig. 3*B*). Again, to verify if this effect was E_2 specific, rats were injected intraperitoneally with 2 mg/kg ICI 182,780 30 min before E_2 subcutaneous administration, and killed over 8 h. The inhibitor was able to fully

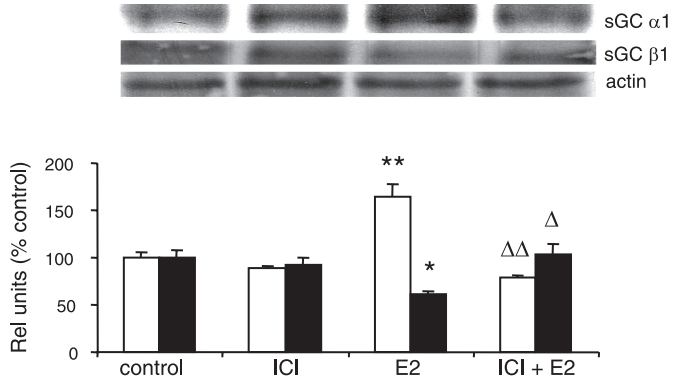

Fig. 2. E_2 actions on sGC protein expression are abolished by pure antiestrogenic ICI 182,780 pretreatment in rat anterior pituitary gland. Adult intact rats at random stages of estrous cycle ($n = 5$ per group) were injected subcutaneous with 2 mg/kg ICI 182,780 (ICI) 30 min before 40 μg/kg body wt E_2 injection and killed after 8 h. *Top*: representative Western blot. *Bottom*: average densitometric values. Bars represent media ± SE of the densitometric values of $sGC\alpha_1$ (open bars) and $sGC\beta_1$ (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. E_2, ANOVA followed by Dunnett's test.

abolish α_{2i} expression increase and had no effect per se on $sGC\alpha_{2i}$ (Fig. 3*C*). Thus the augmented sGCα 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 E_2 on sGC.

sGC subunit expression levels are variable through estrous cycle

Taking into account the acute E_2 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 E_2 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 $sGC\alpha_1$ showed a significant increase through the cycle from proestrus to diestrus (Fig. 4*A*). $sGC\beta_1$ protein levels, which were higher than $sGC\alpha_1$ on the afternoon of proestrus, did not change during estrus but significantly decreased at diestrus. Similar results were observed when $sGC\alpha_1$ 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 ± SE; $n = 3$; male $sGC\alpha_1$: 1.41 ± 0.15 and $sGC\beta_1$: 1.54 ± 0.2; and 14-day ovariectomized $sGC\alpha_1$: 1.43 ± 0.42 and $sGC\beta_1$: 1.61 ± 0.23).

$sGC\alpha_{2i}$ expression levels are variable through the estrous cycle

Bearing in mind our findings showing that $sGC\alpha_1$ expression increases over the cycle in spite of the decrease in cGMP production, the $sGC\alpha_{2i}$ 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. 4*B*). 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 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 E_2 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 E_2 for 6 h, and $sGC\alpha_1$ and β_1 expression was evaluated by Western blot. NO treatment upregulated both subunits in a

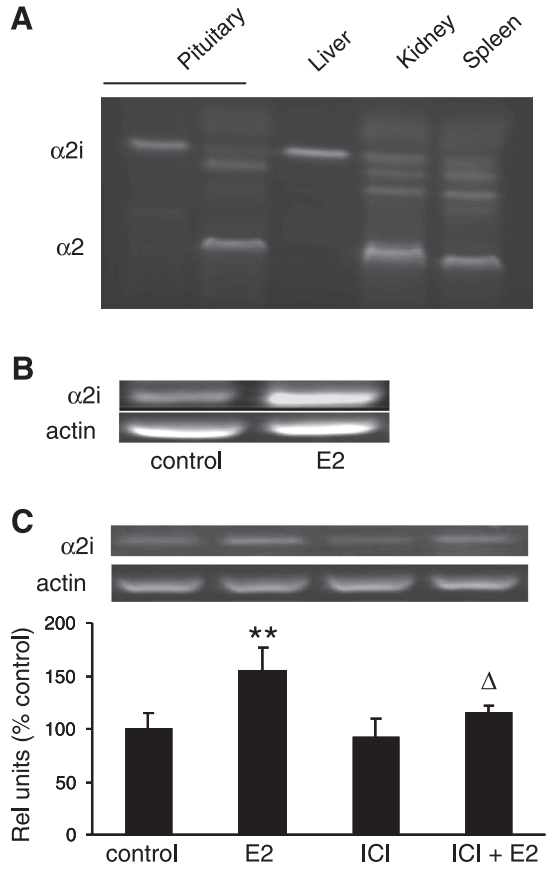

Fig. 3. Other isoforms of sGCα are expressed normally in rat anterior pituitary gland. *A*: representative PCR showing the presence of $sGC\alpha_2$ and α_{2i} mRNAs in anterior pituitary. Liver, kidney, and spleen were used as respective control tissues. *B*: acute E_2 exposition increases the mRNA expression of the dominant negative isoform $sGC\alpha_{2i}$. Animals were injected with 40 μg/kg body wt E_2 and killed after 8 h. *C*: role of ER in $sGC\alpha_{2i}$-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 μg/kg body wt E_2 injection and killed after 8 h. Bars are means ± SE of the densitometric values of $sGC\alpha_i$ mRNA normalized to actin, as percentage of control ($n = 3$). $^*P < 0.05$ vs control; $^{\Delta}P < 0.05$ vs. E_2, ANOVA followed by Tukey's test.

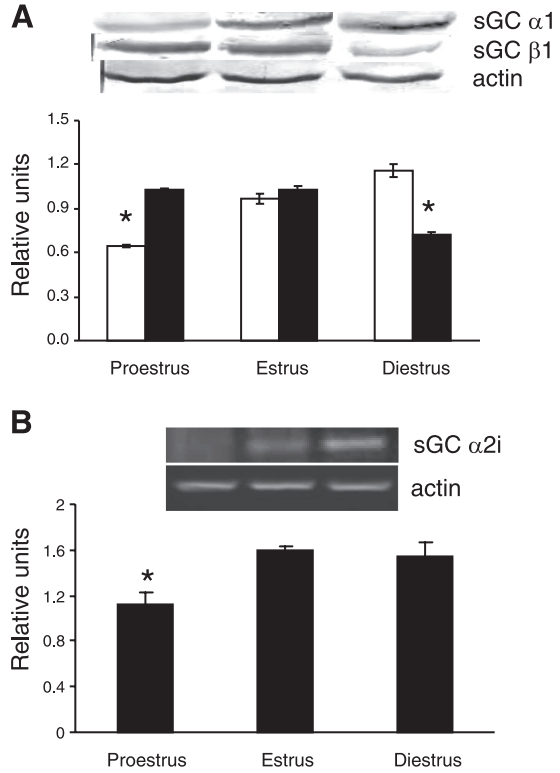

Fig. 4. sGC subunit expression fluctuates over estrous cycle. Rats ($n = 3$ per group) were killed at 1700 on proestrus, estrus, or diestrus. *A*, *top*: a representative Western blot; *bottom*: average densitometric values. Bars are means ± SE of $sGC\alpha_1$ (open bars) and $sGC\beta_1$ (solid bars) protein values normalized to actin ($n = 3$). $^*P < 0.05$ vs. estrus, ANOVA followed by Tukey's test. *B*, *top*: a representative PCR of $sGC\alpha_{2i}$; *bottom*: average densitometric values. Bars are means ± SE of $sGC\alpha_{2i}$ mRNA levels normalized to actin ($n = 3$). $^*P < 0.05$ vs. estrus and diestrus, ANOVA followed by Tukey's test.

similar fashion (Fig. 5*A*) while E_2 treatment increased α_1 but decreased β_1 expression (Fig. 5*B*). In addition, $sGC\alpha_1$ 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; $sGC\alpha_1$: 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 E_2 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 E_2 causes the same effect on sGC subunits in adult and juvenile female animals, suggesting that these effects are distinctive of E_2 and independent of the hormonal fluctuation during the maturation of the reproductive axis. In vivo E_2 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 E_2 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 sGCα 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 E_2 acute treatment enhanced α1 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, $sGC\beta_1$ downregulation would be enough to reduce sGC global activity. However, the higher expression of αGC 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 $sGC\alpha_2$ and $sGC\alpha_{2i}$ isoforms are expressed in anterior pituitary gland and that after E_2 stimulus $sGC\alpha_{2i}$ expression (but not α_2) is augmented. The

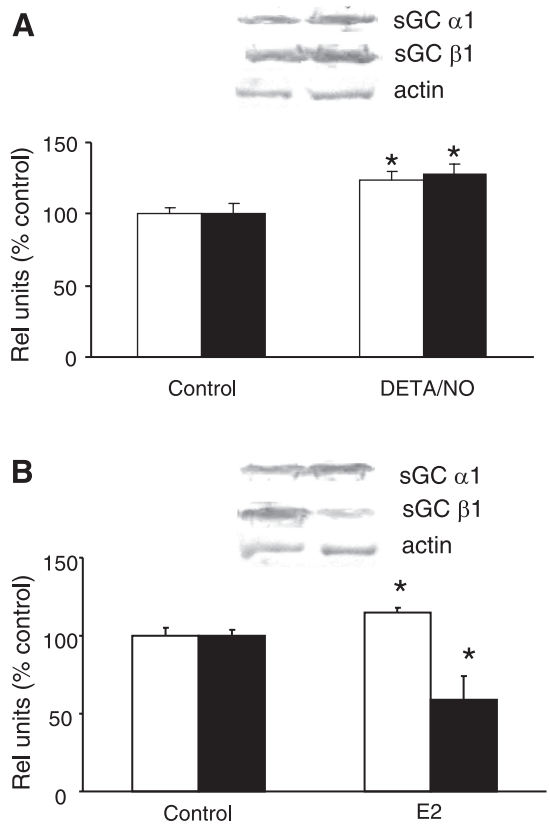

Fig. 5. Short-time nitric oxide exposure upregulated sGC expression, while E_2 acute expression differentially affects sGC subunits in vitro. *A*: primary pituitary cell cultures from adult intact rats at random stages of estrous cycle were incubated with 0.5 mM DETANONOate, a nitric oxide donor, or with culture medium alone (control) for 6 h. *Top*: representative Western blot. *Bottom*: average densitometric values. Bars are media ± SE of $sGC\alpha_1$ (open bars) or $sGC\beta_1$ (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 E_2 or with culture medim alone (control) for 6 h. *Top*: representative Western blot. *Bottom*: average densitometric values. Bars are means ± SE of $sGC\alpha_1$ (open bars) or $sGC\beta_1$ (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 E_2 transient sGC inhibition indicates again that E_2 is acting through multiple pathways. Therefore, our results show that E_2 not only decreases $sGC\beta_1$ expression but also stimulates $sGC\alpha_{2i}$ expression and by these ways participates in sGC activity downregulation.

The estrous cycle is a physiological event in which mainly E_2 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 E_2 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 E_2 level changes and sGC expression pattern. On diestrus, when E_2 levels are rising and NO production is at baseline levels, the expression of $sGC\beta_1$ is the lowest of the entire cycle and, conversely, $sGC\alpha_1$ expression is maximal. Later, on the afternoon of proestrus, when serum E_2 levels have drastically fallen and NO production is high, $sGC\alpha_1$ expression decreases, while a marked increase in $sGC\beta_1$ expression is observed. However, the changes in sGC subunit expression are not identical to that obtained after E_2 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 E_2 levels, beginning on diestrus I, would be enough to cause the differential changes in the sGC subunits on diestrus.

Our results show that E_2 treatment enhances sGCα expression, including an augment in α_{2i}. However, the proportion of α_{2i} vs. α_1 expression is very low. Why does E_2 augment $sGC\alpha_1$ expression if it would not be involved in sGC enzyme constitution? There is some evidence that strongly suggests that the $sGC\alpha_1$ subunit can be individually involved in other processes, independently of cGMP production. In advanced prostate cancer, $sGC\alpha_1$ levels are highly correlated with proliferation (5). E_2 as a mitogenic factor, as well as a proapoptotic stimulus, drives changes in pituitary cell poblation (11, 16, 20). $sGC\alpha_1$ levels and timing of pituitary cell proliferation seem to be correlated. $sGC\alpha_1$ 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 E_2 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 sGCα 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 function, will raise new perspectives and novel pathways to a well-known enzyme.

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