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Neuromodulatory effect of oestradiol in the metabolism of ovarian progesterone and oestradiol during dioestrus II: participation of the superior mesenteric ganglion

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Abstract. The aims of the present study were to determine: (1) whether oestradiol (E_2) in the superior mesenteric ganglion (SMG) modifies the release of ovarian progesterone (P_4), androstenedione (A_2) and E_2, the activity and gene expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-HSD and the expression of P450 aromatase (*Cyp19a1*) and (2) whether any such modifications are related to changes in ovarian nitric oxide (NO) and noradrenaline (NA) levels during dioestrus II. Using an *ex vivo* SMG–ovarian nervous plexus–ovary system, ovarian P_4 release was measured following the addition E_2 plus tamoxifen (Txf) (10^{-6}M) to the ganglion, whereas A_2, E_2, NA and NO were measured following the addition of E_2 alone. Steroids were measured by radioimmunoassay, NA concentrations were determined by HPLC and gene expression was evaluated using reverse transcription–polymerase chain reaction. Oestradiol in the ganglion decreased ovarian P_4, E_2 and NA release, as well as 3β-HSD activity, but increased the release of A_2 and nitrites, as well as the 20α-HSD expression and its activity. No changes were observed in *Cyp19a1* gene expression. The addition of E_2 plus Txf to the ganglion reversed the effects of E_2 alone. The action of oestradiol in SMG favours the beginning of functional luteolysis, due to an increase in NO release and a decrease in NA in the ovary. These results may help elucidate the role of E_2 in hormone-dependent pathologies in women.

Additional keywords: nitric oxide, noradrenaline, ovary, steroidogenesis.

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Introduction

There is ample evidence of the existence of a close interaction between the activity of the peripheral nervous system (PNS) and ovary function at different reproductive stages in the rat (Sosa *et al.* 2000; Casais *et al.* 2006; Vega Orozco *et al.* 2010; Daneri *et al.* 2013; Bronzi *et al.* 2013, 2015).

The mammalian ovary is innervated by two pathways, one via the superior ovarian nerve (SON) and the other through the ovarian nervous plexus (ONP; Klein and Burden 1988*a*). The ONP, which accompanies the ovarian artery to the ovary and enters via the hilum, primarily consists of fibres containing neurotransmitters such as noradrenaline (NA), nitric oxide (NO), substance P (SP) and neuropeptide Y (NPY). These fibres originate in principal neurons located at the superior mesenteric ganglion (SMG; Klein and Burden 1988*a*). The SMG neurons have a variety of putative receptors for certain hormones such as progesterone (P_4), oestradiol (E_2) (Vega Orozco *et al.* 2012) and androstenedione (A_2) (Vallcaneras *et al.* 2009), as well as numerous neurotransmitters (Klein and Burden 1988*b*; Järvi 1989; Sosa *et al.* 2000), which are capable of modulating ovarian steroidogenesis. The ONP principally innervates the musculature of blood vessels, serving an important function in regulating intraovarian blood flow as well as being involved in the regulation of ovarian steroidogenesis (Klein and Burden 1988*a*; Orozco *et al.* 2006; Daneri *et al.* 2013).

Vega Orozco *et al.* (2012) have demonstrated that E_2 in the SMG, through the ONP, is capable of causing variations in NO at the ovarian level, modifying steroidogenesis during oestrus. There is evidence for the existence oestrogen αER and βER receptors in neurons of autonomic ganglia, with the expression

of these receptors varying according to the oestrous cycle, which affects the function of the female reproduction system (Klein and Burden 1988*b*; Järvi 1989; Anesetti *et al.* 2009).

NO is an important paracrine mediator that has a variety of physiological functions. Several lines of evidence suggest that NO is involved in various cycle-dependent ovarian events, such as ovulation and the regulation of luteal function (Van Voorhis *et al.* 1994; Olson *et al.* 1996; Motta *et al.* 2001). It is of note that immunofluorescence studies have demonstrated the presence of the neuronal NO synthase (nNOS) in the sympathetic ganglia (Lars-Gösta *et al.* 1997) and that E_2 is capable of regulating the expression of the gene encoding this enzyme through the αER (Berman *et al.* 1998).

In addition, a single dose of E_2 has been shown to modify expression of tyroxine β-hydroxylase, which is present in the sympathetic ganglia (Shinohara *et al.* 1998). This enzyme participates in the biosynthesis of catecholamines, which are important neurotransmitters in the neural pathways that reach the ovary. Catecholamines modify the activity of sympathetic nerves in the mammalian ovary (Lara *et al.* 2002), leading to changes in steroidogenesis (Bronzi *et al.* 2013) and follicular growth (Aguado and Ojeda 1984; Lara *et. al*. 2002; Rosa-e-Silva *et al.* 2003).

During dioestrus II, the corpus luteum (CL), which is the dominant steroidogenic structure in the ovaries, undergoes functional regression in order for the oestrous cycle to continue. Functional regression, which is followed by structural regression, is evident for a decrease in the production of progesterone by the corpora lutea (Stocco *et al.* 2007). However, the roles of the SMG and E_2 in the ganglion in the mechanisms leading to luteal functional regression during dioestrus II has not yet been elucidated.

Therefore, the main aims of the present study were to determine whether E2 in the SMG, through the ONP, modifies the release of ovarian P_4, A_2 and E_2, the activity and gene expression of 3β-hydroxysteroid dehydrogenase (3β-HSD, *Hsd3b1*) and 20α -hydroxysteroid dehydrogenase (20α-HSD, *Akr1c3*) and the gene expression of P450 aromatase (*Cyp19a1*). In addition, we investigated whether any such changes were related to changes in ovarian NO and NA levels, using an integrated *ex vivo* SMP–ONP–ovary (O) system during dioestrus II.

Materials and methods

Animals

Virgin Holtzman strain adult female rats in dioestrus II, weighing 250 g, were used in all experiments. Rats were kept in a light- and temperature-controlled room (lights on 0700–1900 hours; 24 ± 2°C). Rats had free access to food (Cargill S.A.C.I.) and tap water. Vaginal smears were taken daily, and only rats exhibiting at least two 4-day consecutive oestrous cycles were used. Groups consisted of six rats each. The experiments were performed in duplicate according to the procedures approved in the Universities Federation for Animal Welfare (UFAW) Handbook on the Care and Management of Laboratory Animals (Poole 1999). The experimental protocol was approved by the University of San Luis Animal Care and Use Committee (Protocol no.: B17/04; Ordinance: CD 006/02).

Reagents

Oestradiol valerate (E_2), bovine serum albumin fraction V (BSA), sulfanilamide and *N*-1-naphthyl-ethylenediamine were purchased from Sigma Chemical. Other reagents and chemicals were of analytical grade. 1,2,6,7-[^{3}H]-progesterone (3.959×10^9 Beq mmol^{-1}), 1,2,6,7-[^{3}H]-androst 4-ene-3,17 dione (4.255×10^9 Beq mmol^{-1}) and 17 β-2,4,6,7-[^{3}H]-oestradiol (3.774×10^9 Beq mmol^{-1}) were obtained from New England Nuclear Products.

Surgical and experimental procedures

Extraction of the SMG–ONP–O system

The SMG–ONP–O system was dissected from rats between 1500 and 1600 hours using previous descriptions of the anatomical trajectory of this neural pathway as a guide (Klein and Burden 1988*a*). Briefly, rats were anaesthetised with an intraperitoneal application of Ketamine-xylazine hydrochloride (3/1 relation) and the ex vivo system (SMG–ONP–O) was immediately removed by dissection. Each system consisted of the left ovary, the fibres constituting the ONP, parallel to the ovarian artery, and the SMG, surrounded by some small ganglia. It is important to clarify that the left system was extracted from each rat because the left ovary shows a greater sensitivity to neural stimulation in terms of ovarian steroid release compared with right ovary throughout the oestrous cycle in the rat (Orozco *et al.* 2006; Vega Orozco *et al.* 2010, 2012).

The entire surgical procedure was completed in 1–2 min (Sosa *et al.* 2000). The extracted systems were washed with incubation solution and then placed into a cuvette with two compartments (Sosa *et al.* 2000; Orozco *et al.* 2006). The ganglion was placed in one compartment and the ovary was placed in the other, with the joined by the ONP, which was maintained superfused continuously using work solution. The work solution consisted of Krebs–Ringer bicarbonate buffer, pH 7, containing 0.1 mg mL^{-1} glucose and 0.1 mg mL^{-1} albumin, maintained at 37°C in an atmosphere consisting of 95% O_2 and 5% CO_2.

The SMG–ONP–O system was equilibrated for 15 min with Krebs Ringer Buffer, as described previously (Orozco *et al.* 2006). At the end of the equilibration period (Time 0 of incubation), the buffer was changed in both compartments, and ascorbic acid (0.1 mg mL^{-1} in Krebs'–Ringer) was added as an antioxidant agent (Koh and Hille 1997) to the compartment containing the ganglion, whereas Krebs'–Ringer buffer was added in the compartment containing the ovary. The control group consisted of the SMG–ONP–O system without any treatment. Three experimental groups were established based on the addition of the following to the compartment containing the ganglion: Group 1, E_2 (10^{-6} M); Group 2, tamoxifen (Txf) (10^{-6} M) and Group 3, Txf plus E_2 (10^{-6} M). These experimental groups were used to determine P_4 release in ovary to determine whether Txf acts as an oestrogenic antagonist at the ganglionic level. Group 1 was used only to assess ovarian release of A_2, E_2, nitrites and NA.

The drugs were dissolved in to the desired concentration (10^{-6} M) and volume (2 mL) in Krebs–Ringer buffer plus ascorbic acid. The concentrations used were those that had

exhibited the greatest effects on P_4 release in dose–response curve (Vega Orozco *et al.* 2012).

Samples of the fluid in the ovary compartment (250 μL) were collected after 15, 30, 60 and 120 min incubation and kept at −20°C until determination of P_4, A_2 and E_2 by radioimmunoassay (RIA). For determination of nitrites and NA, the fluid from the chamber in which the ovary was incubated was collected after 120 min incubation, and samples were stored at −80°C until determination of nitrite and NA concentrations using the Griess method and HPLC respectively.

At the end of the incubation period (120 min), the ovaries were frozen and stored at −80°C until determination of 3β-HSD and 20α-HSD activity and gene expression and *Cyp19a1* gene expression.

Determination of P_4, E_2 and A_2

Steroids were measured in duplicate by RIA. The antisera against P_4 were kindly provided by Dr Ricardo Deis (Laboratorio de Reproducción y Lactancia). P_4 is reported as ng mg^{-1} ovary mL^{-1} and the assay sensitivity was <5 ng mL^{-1} P_4. Concentrations of E_2 and A_2 are expressed as pg mg^{-1} ovarian tissue mL^{-1}. The sensitivity of the assays for E_2 and A_2 was <2.2 and <10 pg mL^{-1} respectively. The inter- and intraassay CVs for all assays were $<10.0\%$. The result of each experiment is expressed with regard to values obtained in the corresponding control group.

Enzyme activity

3β-HSD and 20α-HSD activity was measured according to the methods of Kawano *et al.* (1988) with a slight modification. Briefly, the left ovary from each rats was homogenised in 0.7 mL of 0.1 M Tris-HCl and 1 mM EDTA (pH 8) at 0°C with a glass homogenizer, after which the homogenates were centrifuged at 105 000*g* for 60 min at 4°C. The supernatant was used for the assay of 20α-HSD activity. To evaluated 3β-HSD activity, the pellets were resuspended in 0.7 mL of 0.25 M sucrose and centrifuged at 800*g* for 5 min at 4°C. The supernatant was then used in the assay of 3β-HSD activity.

The substrates for 3β-HSD and 20α-HSD were pregnenolone (5 μg) and 20α-hydroxypregn-4en-3-one (12.5 μg) respectively. The activity of both enzymes was assayed spectrophotometrically as the increase in NADH or NADPH in 1 min at 37°C, with values expressed as mU mg^{-1} protein min^{-1}. Protein concentrations were determined according to the method of Lowry *et al.* (1951) with BSA as the standard.

RNA isolation and reverse transcription–polymerase chain reaction analysis

Once the ovaries had been defrosted, total RNA was extracted using the TRIzol reagent method (Invitrogen Life Technologies), according to the manufacturer's instructions for RNA extraction (Chomczynski 1993). Briefly, 2 μg T total RNA was reverse transcribed at 37°C using random primers and M-MLV Reverse Transcriptase (Promega) in a reaction volume of 26 μL.

Fragments coding for *Hsd3b3*, *Akr1c3* and *Cyp19a1* were amplified by polymerase chain reaction (PCR) in 50 μL reaction solution containing 0.2 mM dNTPs, 1.5 mM MgCl, 1.25 U Taq polymerase, 50 pmol of each rat-specific oligonucleotide primer and reverse transcription (RT)-generated cDNA (one-fifth of the RT reaction). The sequences of the primers used in the present study are as reported previously (Casais *et al.* 2012; Bronzi *et al.* 2013). cDNA was amplified using a thermal cycler (MyCycler; BioRad Laboratories). The reaction products were electrophoresed on 2% agarose gels, visualised using Gel Red and examined under ultraviolet transillumination. Band intensities of RT-PCR products were quantified using ImageJ (http://rsb.info.nih.gov/ij/, accessed 17 March 2017). Relative mRNA levels are expressed as the ratio between the signal intensity of the target genes and that of the housekeeping *16S* ribosomal RNA (*S*16).

Nitrite assay

Nitrite, a water-soluble metabolite of NO, was measured spectrophotometrically by the Griess method, with concentrations expressed as nmol mg^{-1} ovary mL^{-1} (Egami and Taniguchi 1974). To determine nitrite concentrations, a 50-μL aliquot of the fluid bathing the ovary was immediately mixed with Griess reagent (sulfanilamide with *N*-1-naphthyl-ethylenediamine/HCl). After 10 min incubation at room temperature, the absorbance of the mixture was determined at 540 nm, and the amount of nitrite (nmol) was read off a standard curve. The assay sensitivity was <2.5 nmol mL^{-1}. The intraassay coefficients of variation for all assays was $<10.0\%$.

HPLC analysis of catecholamines

NA was the catecholamine measured using a previously reported method (Einsenhofer *et al.* 1986). Briefly, 20-μL aliquots of the fluid from the cuvette containing the ovary were partially purified by batch alumina extraction and separated by reverse-phase HPLC using a 4.6 × 250 mm Bridge × C18 column (Waters). Recovery through the alumina extraction step averaged 70–80% for catecholamines. Catechol concentrations in each sample were corrected for recovery of the internal standard dihydroxybenzylamine. The detection limit of the assay was approximately 15 pg per volume assayed for each catechol. The electrochemical response was linear ($r = 0.99$) for amounts of NA ranging from 50 to 2000 pg. The interassay variation coefficients were 15% and the intra-assay variation coefficients was 10% for NA.

Statistical analysis

All data are presented as the mean ± s.e.m. for each group of six rats. The significance of differences between two groups was analysed using Student's *t*-test. For multiple comparisons made the along the time of incubation, repeated-measures analysis of variance (ANOVA) followed by Tukey's test was used. A difference was considered to be statistically significant at $P < 0.05$ (Snedecor and Cochram 1976).

Results

Effects of E_2 and Txf on ovarian P_4 release during dioestrus II

Because of the importance of E_2 in the modulation of the activity of sympathetic neurons, we stimulated the SMG with E_2 (10^{-6} M) and investigated whether this steroid able to modulate ovarian P_4

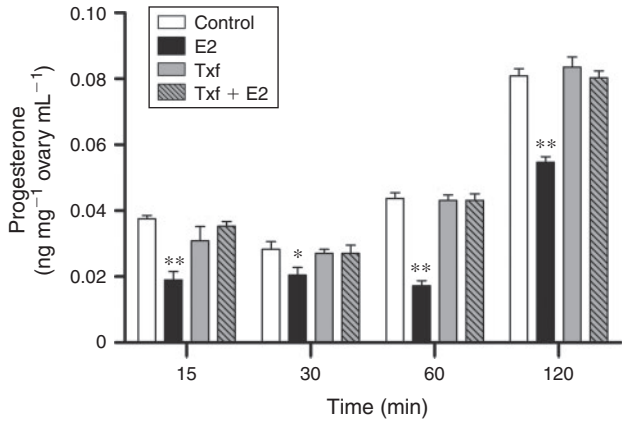

Fig. 1. Effects of the addition of 10^{-6} M oestradiol (E2) and 10^{-6} M tamoxifen (Txf), alone and in combination, to the superior mesenteric ganglion (SMG) on ovarian progesterone release in the rat in the SMG–ovarian nervous plexus–ovary system during dioestrus II. The systems were incubated in Krebs'–Ringer buffer, plus ascorbic acid (0.1 mg mL^{-1} in Krebs–Ringer), at 37°C in an atmosphere of 95% O2 and 5% CO_2 for 120 min without any treatment (control) or after the addition of E2 and Txf. Data are the mean ± s.e.m. ($n = 6$ rats per group). *$P < 0.05$, **$P < 0.001$ compared with control (one-way ANOVA followed by Tukey's test).

release. Addition of E_2 to the ganglion compartment significantly decreased ovarian P_4 release after 15, 30, 60 and 120 min (Fig. 1). However, the addition of Txf alone or Txf plus E_2 to the ganglion compartment had no effect on ovarian P_4 release compared with control (Fig. 1).

Effects of E_2 on ovarian 3β-HSD and 20α-HSD activity and gene expression during dioestrus II

The addition of E_2 to the ganglion compartment significantly ($P < 0.001$) decreased 3β-HSD activity after 120 min incubation, whereas there was no change in *Hsd3b3* gene expression compared with control (Fig. 2*a*, *c*).

Moreover, the addition of E_2 to the ganglion compartment significantly ($P < 0.001$) increased 20α-HSD activity and *Akr1c3* gene expression compared with control (Fig. 2*b*, *d*).

Effects of E_2 on ovarian A_2 and E_2 release, and Cyp19a1 gene expression

The addition of E_2 to the ganglion compartment significantly ($P < 0.001$) increased ovarian A_2 release after 30, 60 and 120 min incubation (Fig. 3*a*). Moreover, the addition of E_2 to the ganglion compartment significantly ($P < 0.001$) decreased ovarian E_2 release at time points during incubation compared with control (Fig. 3*b*). However, there E_2 had no effect on Cyp19a1 gene expression compared with control (Fig. 3*c*).

Effects of E_2 on ovarian nitrites and noradrenaline release

The addition of E_2 to the ganglion compartment significantly ($P < 0.001$) increased ovarian nitrites after 30, 60 and 120 min incubation (Fig. 4*a*), whereas significant decreases in NA release were seen after 30, 60 and 120 min compared with control (Fig. 4*b*).

Discussion

Oestrogens play an important role in female reproduction. A close relationship between oestrogens, sympathetic and sensory neurons and ovarian function has been reported in the rat.

In previous studies, Anesetti *et al.* (2009) and Vega Orozco *et al.* (2012) found that neurons located in the coeliac ganglion (CG) and SMG contain two intracellular oestrogen receptor subtypes, namely αER and βER, which have well-known roles as ligand-activated transcription factors. However, the effect of oestrogens can also be mediated by non-classical membrane-bound receptors, as has been observed in several tissues, for example via activation of G-protein-coupled estrogen receptor 1 (GPER), also known as G protein-coupled receptor 30 (GPR30), in which case the effects occur within seconds or minutes after the addition of E_2 in different experimental models (Weihua *et al.* 2003; Song *et al.* 2005). Studies performed in ER-knockout ($ER^{-/-}$) mice have shown changes in reproductive function, with modifications in ovarian folliculogenesis and, as a consequence, in ovulation and CL formation (Couse *et al.* 2003).

Previous studies suggest that E_2 may be the hormonal key regulating luteal apoptosis in reproductive processes (Stocco *et al.* 2007). Vega Orozco *et al.* (2012) demonstrated that E_2 stimulation of the SMG during oestrus activates αER in the SMG, and that E_2 increases ovarian P_4 release, thus participating in CL formation.

The results in the present study are opposite to those obtained during oestrus, because ganglionic oestrogenic stimulation inhibited the release of ovarian P_4 at all time points studied. This decrease in ovarian P_4 after ganglionic stimulation occurred after 15 min incubation, which makes it evident that E_2 may be acting at the ganglion level through receptors associated with non-genomic mechanisms, such as αER, or possibly through rapid membrane-associated receptors, such as GPR30, as has already been demonstrated in numerous peripheral organs (Hazell *et al.* 2009; Mani *et al.* 2012).

Previous studies have demonstrated that P_4 production by the CL in dioestrus is more sensitive to neural stimuli than to gonadotropins (De Bortoli *et al.* 1998). This suggests that variations in P_4 levels in the ovary may be caused by the release of numerous neurotransmitters from the ONP to the ovary as NO needed to start the new cycle.

The increase in NO observed in the present study may originate in the SMG, from nNOS, following neural oestrogen stimulation (Delgado *et al.* 2004). This could lead to the release of various neurotransmitters from the ganglion to the ovary that can affect steroidogenesis, as reported previously (Delgado *et al.* 2004; Casais *et al.* 2006).

It is important to note that NO may play a crucial role in the first steps of functional luteolysis by regulating luteal blood flow (Fridén *et al.* 2000; Korzekwa *et al.* 2006; Shirasuna 2010). There is evidence for the presence of endothelial (e) NOS not only in granulosa luteal cells, but also in the endothelial cells near the functional CL. Van Voorhis *et al.* (1994) have shown that elevated levels of E_2 increase eNOS expression in the rat ovary. Therefore, in the present study, the increase in NO may be due to the fact that E_2 (in the SMG, via the ONP) stimulates

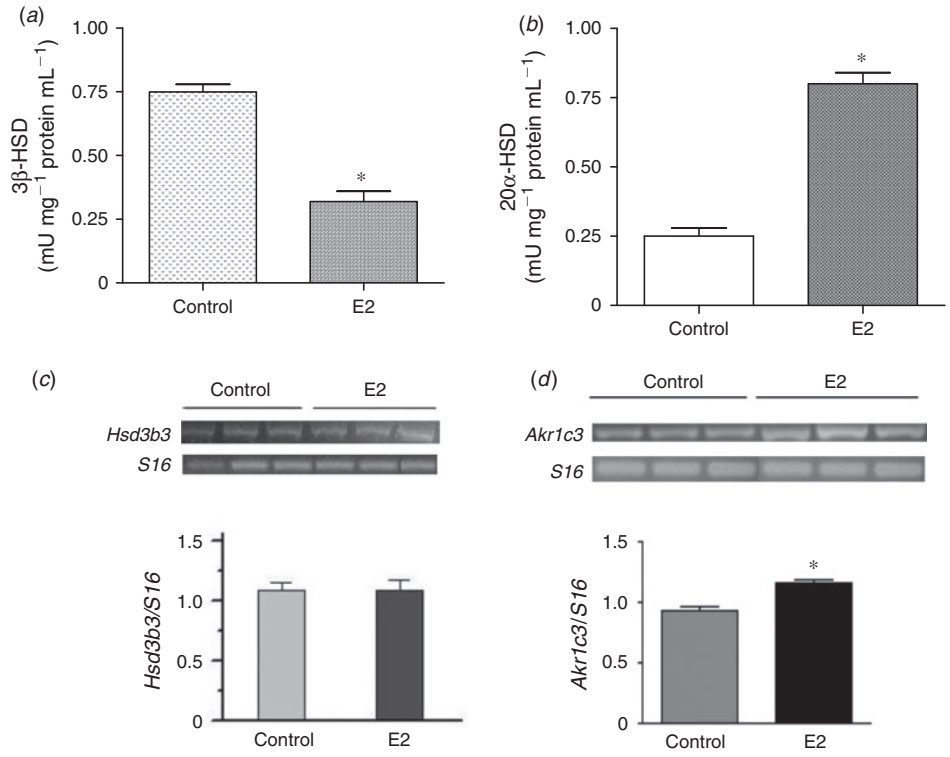

Fig. 2. Effects of the addition of 10^{-6} M oestradiol (E_2) to the superior mesenteric ganglion (SMG) compartment in the rat in the SMG–ovarian nervous plexus–ovary system on ovarian (*a*) 3β-hydroxysteroid dehydrogenase (HSD) and (*b*) 20α-HSD activity, and relative expression of genes encoding (*c*) *Hsd3b3* and (*d*) *Akr1c3* during dioestrus II. Enzyme activity and relative gene expression were determined after 120 min incubation with E_2. *16 S* ribosomal RNA (S16) was used as the housekeeping gene. Gel photographs were quantified using ImageJ (National Institutes of Health) and relative gene expression is given in arbitrary units. Data are the mean ± s.e.m. ($n = 3$). *$P < 0.001$ compared with control (Student's *t*-test).

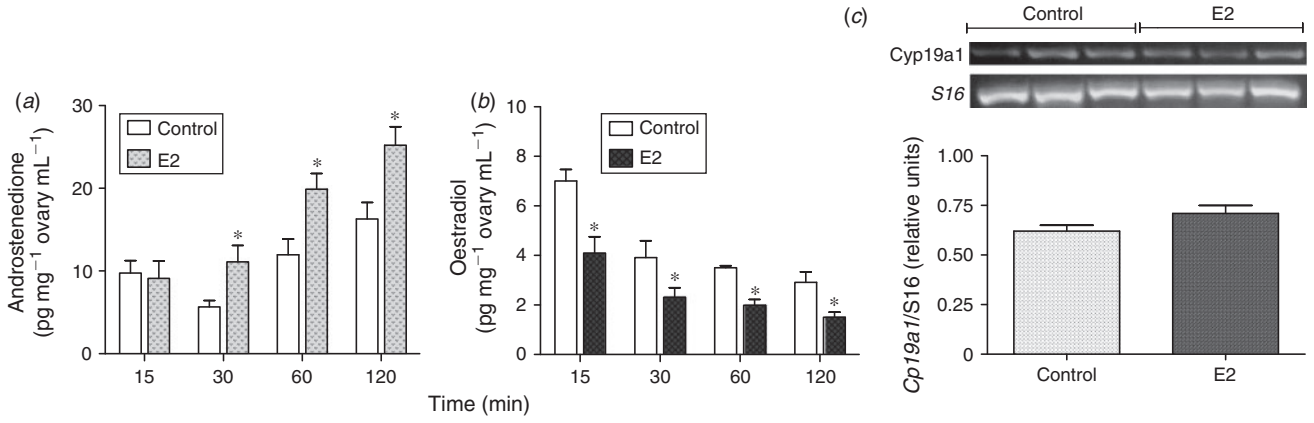

Fig. 3. Effects of the addition of 10^{-6} M oestradiol (E_2) to the superior mesenteric ganglion (SMG) compartment in the rat in the SMG–ovarian nervous plexus–ovary system on ovarian (*a*) androstenedione and (*b*) oestradiol release, as well as (*c*) Cyp19a1 mRNA gene expression during dioestrus II. *16 S* ribosomal RNA (*S16)* was used as the housekeeping gene. Determinations were made after 120 min incubation. Data are the mean ± s.e.m. of six rats in each group. Experiments were performed in duplicate. *$P < 0.001$ compared with control (Student's *t*-test).

eNOS and thus increases ovarian NO levels, affecting the activity of steroidogenic cells (Jaroszewski and Hansel 2000). The inhibitory action of NO, through the ONP, on progesterone syntheses has been demonstrated here.

It is worth noting that in accordance with the decrease in ovarian P_4 release, there was a decrease in the activity of 3β-HSD (the enzyme responsible for P_4 synthesis) and an increase in the activity and gene expression of 20α-HSD, which degrades P_4.

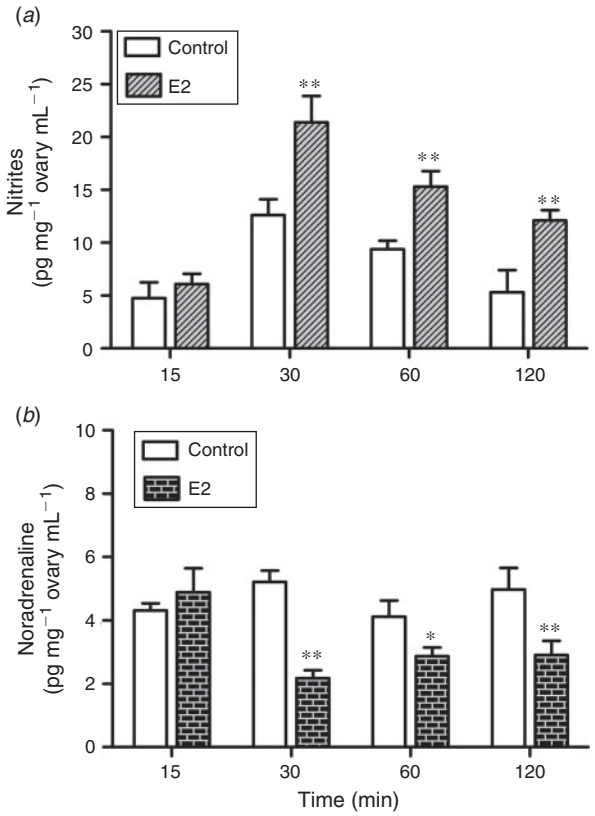

Fig. 4. Effects of 10^{-6} M oestradiol (E2) on ovarian (*a*) nitrites and (*b*) noradrenaline release into the incubation medium in the ovarian compartment. The superior mesenteric ganglion (SMG)–ovarian nervous plexus–ovary system obtained from rats during dioestrus II was incubated in Krebs'–Ringer buffer plus ascorbic acid ($0.1\,mg\,mL^{-1}$) at 37°C in an atmosphere of 95% O2 and 5% CO_2 for 120 min without (control) or after the addition of E2 to the SMG compartment. Data are the mean ± s.e.m. of six rats in each group. $^*P < 0.01$, $^{**}P < 0.001$ compared with control (one-way ANOVA followed by Tukey's test).

Stocco *et al.* (2001) demonstrated that a decrease in ovarian P_4 levels is an essential step towards the induction of 20α-HSD expression at the end of pregnancy. These authors demonstrated that an increase of 20α-HSD is considered an indicator of functional luteolysis related to a reduced capacity of the CL to produce P_4, leading to the death of luteal cells (Stocco *et al.* 2001).

In order to verify the effect of E_2 on ERs, the SMG was stimulated with and without Txf, an ER antagonist.

Certain compounds, such as tamoxifen citrate and the phytoestrogens, have differential actions that are tissue specific and depend on ER subtypes, which may explain their different effects (Weihua *et al.* 2003; Song *et al.* 2005). When Txf alone or Txf plus E_2 were added to the ganglion compartment, no changes were observed in the release of ovarian P_4 compared with the control group at any time of incubation, which indicates that ERs are present in the ganglion and that Txf is capable of blocking the effects of E_2 on P_4 release.

The release of A_2 and E_2 was also evaluated in the ovarian incubation fluid, with increases seen in A_2 in ovary after 30, 60 and 120 min incubation, the latter coinciding with the maximum decrease in E_2. It has been demonstrated that the enzyme responsible for the synthesis of E_2 from A_2 is cytochrome P_{450} aromatase. In this case, the gene expression of *Cyp19a1* did not exhibit any changes compared with control at any of the time points studied. It is important to emphasise that FSH and LH are well-known regulators of expression in rat granulosa cells (Hickey *et al.* 1988). The results in the present study may be due to the absence of the gonadotropin in the *ex vivo* system used or to the short incubation times.

Conversely, although no changes were observed in the expression of the *Cyp19a1* gene, there was a significant decrease in ovarian E_2 levels following ganglionic stimulation, which could be due to a decrease in aromatase activity. *In vitro* studies have demonstrated that NO may inhibit aromatase activity both by decreasing Cyp19a1 mRNA levels and by a direct inhibitory effect on the enzyme (Johnson *et al.* 1999; Banerjee *et al.* 2012). The results of the present study are in agreement with these results, because NO increased but E_2 decreased P450 aromatase activity, without changing gene expression, at least at the time points investigated here.

In the present study, the addition of E_2 to the SMG compartment decreased ovarian P_4 and E_2 release at all incubation times, favouring functional luteolysis and thus facilitating the continuity of the oestrous cycle. However, Bronzi *et al.* (2013) have demonstrated that E_2 in the CG does not have the same effect on ovarian P_4 release, but rather protect the CL against ovarian apoptotic mechanisms that occur in dioestrus II.

These differential responses of the SMG and CG to oestrogenic stimulation may be due to the fact that the ganglia communicate with each through nerve connections (Lawrence and Burden 1980). The CG is composed of principal neurons whose axons form the superior ovarian nerve (SON) from which certain fibres emerge and make 'synaptic contact' with principal neurons in the SMG, thereby modulating the post-ganglionic outflow of these post-ganglionic neurons (Lawrence and Burden 1980).

It is also possible that the different effects of E_2 observed in the SMG and CG could be due to the different intraovarian structures that innervate the nerve pathways (Lawrence and Burden 1980; Erickson *et al.* 1985).

It is also important to note that different neurotransmitters could be released from the ONP to the ovary following stimulation of the ganglion, including NPY and gonadotrophin-releasing hormone (GnRH), and together may modulate the final response in the ovary. Together, these results demonstrate the complexity of the events occurring in the ovary–ganglion structure and, consequently, the many factors that may participate simultaneously in a determined effect.

In addition to NO, catecholamines play a critical role in ovarian physiology. In fact, it has been demonstrated that both NA and NO play a role in maintaining the balance between luteal development and regression (Skarzynski and Okuda 2000). In the present study, the release of NA at the ovarian level following E2 stimulation of the SMG decreased after 30, 60 and 120 min incubation. The maximum fall observed at 30 min coincides with the maximum peak in NO, which suggests that both neurotransmitters regulate each other, as observed by others (Skarzynski and Okuda 2000; Skarzynski *et al.* 2000).

Although the presence of catecholamines is necessary for the normal development of the CL, the results of the present study indicate that NO may participate, through the ONP, in its regression by regulating NA content in the ovary, as has been demonstrated previously (Skarzynski *et al.* 2000). This effect in the ovary may lead to the initiation of functional luteolysis of the CL at the end of the oestrous cycle, which is in accordance with results obtained for the initiation of delivery at the end of pregnancy (Casais *et al.* 2012).

As a whole, the results reported herein demonstrate that E_2 stimulation of the SMG decreases levels of P_4 and E_2 at the times studied and that NO appears to be involved in this effect.

These findings demonstrate the complexity of the sympathetic ganglia and the numerous factors that participate in the regulation of ovarian function.

Finally, basic knowledge of the effects of E_2 on the sympathetic neurons is significant because this steroid can participate in the development and maintenance of hormone-dependent pathologies affecting women's health.

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