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Involvement of the oestrogenic receptors in superior mesenteric ganglion on the ovarian steroidogenesis in rat

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Abstract

Oestradiol (E_2) is a key hormone in the regulation of reproductive processes. The aims of this work were a) to examine the distributions of oestrogen receptor α (ERα) and ERβ in the neurons of the superior mesenteric ganglion (SMG) in the oestrus stage by immunohistochemistry, b) to demonstrate whether E_2 in the SMG modifies progesterone (P_4), androstenedione (A_2) and nitrite release in the ovarian compartment on oestrus day and c) to demonstrate whether E_2 in the ganglion modifies the activity and gene expression in the ovary of the steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-hydroxysteroid dehydrogenase (20α-HSD). The *ex vivo* SMG–ovarian nervous plexus–ovary system was used. E_2, tamoxifen (Txf) and E_2 plus Txf were added in the ganglion to measure ovarian P_4 release, while E_2 alone was added to measure ovarian A_2 and nitrites release. Immunohistochemistry revealed cytoplasmic ERα immunoreactivity only in the neural somas in the SMG. E_2 increased ovarian P_4 and A_2 release at 15, 30 and 60 min but decreased nitrites. The activity and gene expression of 3β-HSD increased, while the activity and gene expression of 20α-HSD did not show changes with respect to the control. Txf in the ganglion diminished P_4 release only at 60 min. E_2 plus Txf in the ganglion reverted the effect of E_2 alone and the inhibitory effect of Txf. The results of this study demonstrate that ERα activation in the SMG has an impact on ovarian steroidogenesis in rats, thus providing evidence for the critical role of peripheral system neurons in the control of ovarian functions under normal and pathological conditions.

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Introduction

There is ample evidence demonstrating a functional interaction between the peripheral nervous system and the reproductive system (Sosa *et al*. 2000, 2004, Casais *et al*. 2006). The ovary receives sympathetic innervation from the prevertebral ganglia by two routes: the ovarian nervous plexus (ONP) and the superior ovarian nerve (SON). The ONP fibres originate mostly in the neurons of the superior mesenteric ganglion (SMG; Baljet & Drukker 1979, Klein & Burden 1988*a*) and accompany the ovary artery in its entrance to the hilum. This pathway not only innervates the blood vessels but also the veins that form a net around the theca cells (Lawrence & Burden 1980). The SMG is included in the sympathetic prevertebral chain; it is constituted by principal neurons and interneurons (Eränkö 1978, Prud'Homme *et al*. 1999) and it has a profuse capillary plexus that constitutes a microcirculation among the different ganglionic structures. The sympathetic ganglia possess a great variety of specific receptors and neurotransmitters, such as catecholamines (Klein & Burden 1988*a*) and neuropeptides (Dalsgaard *et al*. 1982). These ganglia are active points of concurrency for hormone signals, as revealed by the metabolic changes induced by steroids, as well as by the existence of putative receptors for some hormones such as oestradiol (E_2; Cardinali *et al*. 1983).

Oestrogens are important for the development of sexual phenotype and differentiation of the central nervous system (CNS). They play crucial roles in the development of the pituitary gonadal axis, ovarian folliculogenesis, ovulation and implantation, thereby acting on peripheral organs and sympathetic neurons associated with functions related to reproduction (Anesetti *et al*. 2009). Zoubina & Smith (2003) demonstrated that a small number of uterine-projecting neurons located in the SMG and other sympathetic ganglia expressed oestrogen receptors (ERs). Their

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actions are mediated through two distinct receptors, ERα and ERβ, which bind to specific DNA sequences regulating transcription. ERα is also localised in the plasma membrane and may help to elucidate the mechanisms through which rapid, 'non-genomic' oestrogen signalling occurs (Deecher *et al.* 2003, Doolan & Harvey 2003, Wang *et al.* 2005). ERs are regulated in a tissue- and/or cell-type specific manner and E_2 itself seems to play an important role in this regulation (Weihua *et al.* 2003).

A simple dose of E_2 valerate can modify the levels of expression of the enzyme tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, which induces changes in the nervous sympathetic activity in the ovary of mammals (Shinohara *et al.* 1998, Lara *et al.* 2000), provoking alterations in the follicular growth that lead to the development of polycystic ovaries (Lara *et al.* 2002, Rosa *et al.* 2003).

This enzyme, together with dopamine β-hydroxylase, which also participates in the biosynthesis pathway of catecholamines, colocalises with immunoreactive neuropeptides and choline acetyltransferase, the enzyme for the acetylcholine synthesis pathway (Lars-Gösta *et al.* 1997).

Indirect immunofluorescence studies have demonstrated the presence of the isoenzyme neuronal nitric oxide synthase (nNOS) in the SMG (Quinson *et al.* 2001). Berman *et al.* (1998) have demonstrated that ERα is predominantly expressed by nitric oxide synthase (NOS)-positive in parasympathetic pelvic ganglion neurons. There is evidence that NO decreases the steroidogenesis in different experimental schemes (Olson *et al.* 1996, Snyder *et al.* 1996), causing an inhibition of the enzymatic activities and gene expression of steroid synthesis-limiting enzymes such as 3β-hydroxysteroid dehydrogenase (3β-HSD) in bovine luteal cells (Rekawiecki *et al.* 2005). Vega Orozco *et al.* (2006) have demonstrated in an *ex vivo* system that the ganglionic cholinergic stimulus modifies the ovarian steroids and NO release in the oestrus stage.

With these antecedents in mind and considering the neural influence from the SMG through the ONP on ovarian steroidogenesis, the aims of this work were a) to examine the distributions of ERα and ERβ in neurons of the SMG during the oestrus (E) stage by immunohistochemistry, b) to demonstrate whether E_2 in the SMG modifies progesterone (P_4), androstenedione (A_2) and nitrite (NO) release in the ovarian compartment on oestrus day and c) to demonstrate whether E_2 in the ganglion modifies the activity and gene expression in the ovary of the steroidogenic enzymes 3β-HSD and 20α-HSD.

Results

Localisation of ERs in the SMG on oestrus day by immunohistochemistry

The histological study of the SMG showed a thin capsule of connective tissue and the parenchyma exhibited several neuronal profiles (Fig. 1A). ERα immunoreactivity was observed in most neuronal somas of the SMG, and its pattern was cytoplasmic and heterogeneous (Fig. 1B). Uterine control sections displayed the classical nuclear localisation of this receptor. ERβ immunoreactivity was not observed in neuronal somas (Fig. 1C). Figure 1D shows a high magnification of Fig. 1C (40×). Rat ovary was used as the positive control for ERβ (Fig. 1D′). No positive structures or cells were observed in the SMG sections when the primary antiserum was not added (negative control; Fig. 1D″).

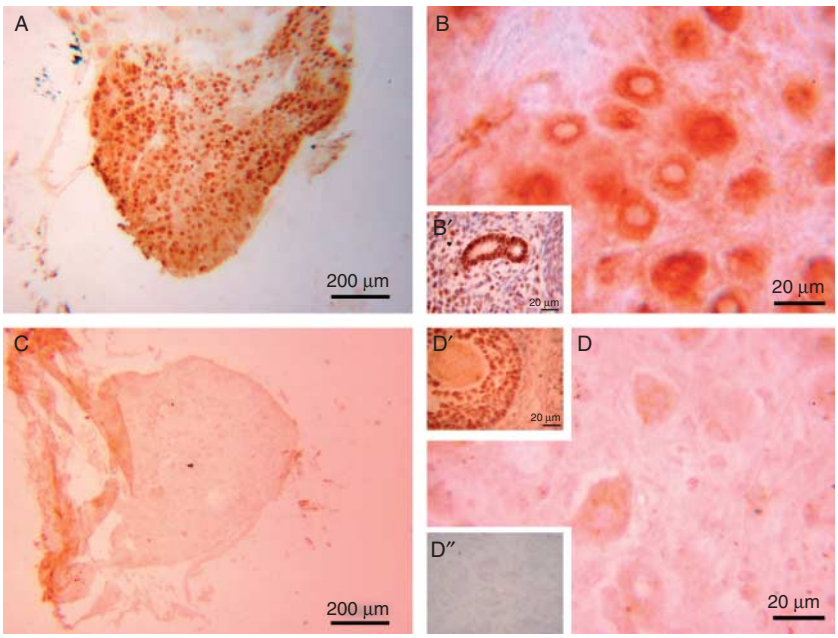

Figure 1 (A–D) (A) Low magnification of the SMG showing that almost all neurons displayed immunoreactivity for ERα (4×). (B) High magnification of A (40×). Note the extensive immunoreactivity restricted to cytoplasm. No nuclear signal was detected. (BB′) Inset of positive control for ERα in rat uterus showing intense signal in glandular and stromal endometrial cells (40×). (C) Low magnification of the SMG showing absence of ERβ reactivity (4×). (D) High magnification of C (40×). (D′) Inset of positive control for ERβ in rat ovary (40×). (D″) Inset of negative control for ERβ in rat SMG.

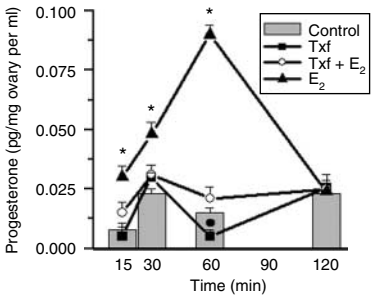

Figure 2 Effect of oestradiol (E_2) and tamoxifen (Txf) in the superior mesenteric ganglion on the ovarian progesterone release in the superior mesenteric ganglion–ovarian nervous plexus–ovary system removed from oestrus day rat. The systems were incubated in a Krebs–Ringer buffer, plus ascorbic acid (0.1 mg/ml in Krebs–Ringer) solution, at 37 °C in an atmosphere of 95% O_2 to 5% CO_2 for 120 min without (control) and with the addition of E_2 (10^{-6} mol/l), Txf (10^{-6} mol/l) and E_2 plus Txf in the ganglion compartment (experimental group). E_2, E_2 valerate. Values are the mean ± S.E.M. of six animals per experimental group. Columns with * and • indicate statistical significance of $^*P<0.001$ and $^\bullet P<0.05$, respectively, compared with the control group (Student's *t*-test, ANOVA, Duncan's test).

Ganglionic effect of E_2 and tamoxifen on ovarian P_4 release on oestrus

To evaluate whether the presence of E_2 and its antagonist in the ganglion compartment modulated the release of ovarian P_4 in the *ex vivo* system and considering that this steroid has proved to be the most sensitive steroid to neural influence in *in vitro* studies, E_2 (10^{-6} mol/l), tamoxifen (Txf) only and E_2 plus Txf were added in the SMG. Txf was used since this antagonist is specific for ERα (Sun *et al*. 2010).

E_2 in the ganglion increased ovarian P_4 release at 15 min (0.008 ± 0.001 vs 0.03 ± 0.001), 30 min (0.023 ± 0.002 vs 0.048 ± 0.001) and 60 min (0.015 ± 0.006 vs 0.09 ± 0.004; $^*P<0.001$), whereas Txf alone decreased P_4 release only at 60 min (0.015 ± 0.002 vs 0.005 ± 0.001; $^\bullet P<0.05$) with respect to the control group. The addition of E_2 (10^{-6} mol/l) plus Txf in the ganglion did not show changes in ovarian P_4 release in comparison with the control group (Fig. 2).

Effect of E_2 in the ganglion on the enzymatic activity and gene expression of the ovarian 3β-HSD and 20α-HSD steroidogenic enzymes on oestrus

To evaluate whether the addition of E_2 in the mesenteric ganglion modified the activity and gene expression of the P_4 synthesis enzymes in the oestrus stage, the ovaries of the system were obtained after 120 min of incubation.

P_4 in the ganglion increased the ovarian enzymatic activity and gene expression of 3β-HSD at 120 min of incubation ($^*P<0.001$; Figs 3a and 4a), whereas the activity and gene expression of 20α-HSD did not show significant changes at 120 min with respect to the control (Figs 3b and 4b).

Effect of E_2 in ganglion on ovarian A_2 release on oestrus

Considering that A_2 is synthesised by the ovarian interstitial cells and that ONP innervated these structures, A_2 release in the ovarian compartment by stimulation with E_2 at the ganglionic level was assessed.

E_2 in the ganglion increased the release of A_2 in the ovarian compartment at 15 min (0.024 ± 0.01 vs 0.354 ± 0.01), 30 min (0.595 ± 0.01 vs 0.92 ± 0.01; $^*P<0.001$) and 60 min (0.42 ± 0.01 vs 0.98 ± 0.01; $^*P<0.001$; Fig. 5).

Effect of E_2 in the ganglion on ovarian nitrite release on oestrus

The modulation of the release of nitrites by neural stimulus and their importance in the reproductive function has been demonstrated in our research group; therefore, we attempted to determine whether the addition of E_2 in the ganglion compartment modifies nitrite release on the ovarian compartment on oestrus day.

E_2 caused a significant decrease in nitrite release at all incubation times in comparison with the control group ($^*P<0.001$; Fig. 6).

Discussion

Given the importance of the neural regulation from the peripheral nervous system on ovarian physiology (Sosa *et al*. 2000, Casais *et al*. 2006, Vega Orozco *et al*. 2010) and taking into account that E_2 acts on peripheral organs and on the plasticity of sympathetic neurons associated with reproduction (Anesetti *et al*. 2009), the general aim of this work was to examine the distributions of ERα and ERβ in neurons of the SMG and to evaluate the importance of these receptors in ovarian steroidogenesis and their possible relation with NO release.

In this work, ERα was typified by immunohistochemistry in the SMG, but ERβ was not detected probably due to the fact that receptors of this type have been

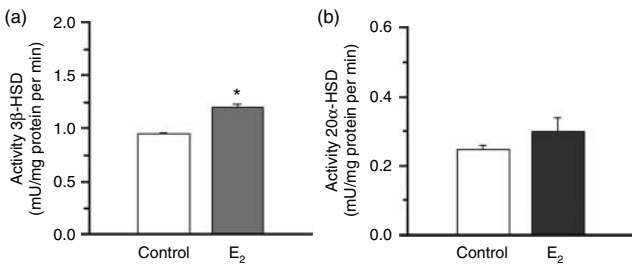

Figure 3 Effect of oestradiol (E_2) in the ganglion compartment on the enzymatic activity of 3β-HSD (a) and 20α-HSD (b) in ovaries extracted from the superior mesenteric ganglion–ovarian nervous plexus–ovary system with and without the addition of E_2 (10^{-6} mol/l) in the mesenteric ganglion at 120 min of incubation on oestrus day. E_2, E_2 valerate. Values are the mean ± S.E.M. of six animals in two experiments. Columns with *indicate statistical significance of $^*P<0.001$ compared with the control group (Student's *t*-test; ANOVA, Duncan's test).

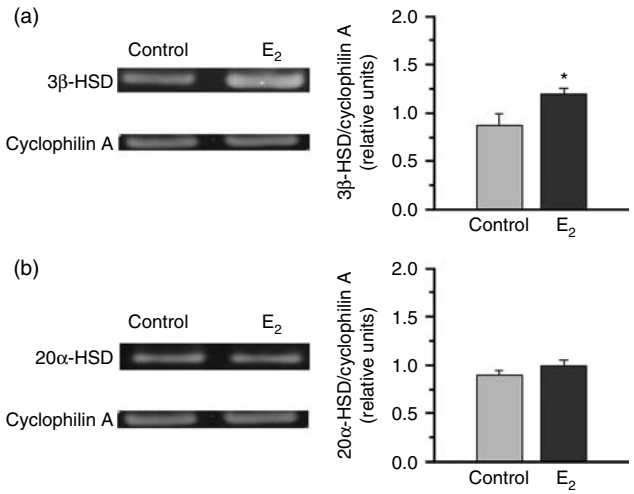

Figure 4 Ganglionic effect of oestradiol on mRNA abundances of enzymes 3β-HSD (a) and 20α-HSD (b) obtained from rat ovary previously incubated in the superior mesenteric ganglion–ovarian nervous plexus–ovary system on oestrus day. Measurement by RT-PCR of the expression of 3β-HSD and cyclophilin A. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products. Expression of 3β-HSD relative to cyclophilin A. The gel photographs were quantified using Image J (NIH, Bethesda, MA, USA) and expressed as arbitrary units. Results are expressed as the mean±S.E.M. ($n=3$). *$P<0.001$.

fundamentally found in prepubertal (Shinohara *et al.* 2000, Anesetti *et al.* 2009) and adult rats in other cycle stages (Zoubina & Smith 2002). The results obtained in this work differ from those reported by Zoubina & Smith (2002), who found, in a different experimental scheme, that the vast majority of sympathetic neurons express ERβ immunoreactive protein, whereas a smaller, presumably overlapping subset expresses the ERα. Papka *et al.* (2001) found that both receptors could potentially be affected by changes in endogenous oestrogen levels, which could explain why ERβ was not found in our study. Our findings showed that in SMG neurons, ERα was mainly distributed uniformly throughout the neuronal cytoplasm on oestrus day, but it was not observed in satellite or Schwann cells, in agreement with the findings reported by Anesetti *et al.* (2009) on lumbar paravertebral and prevertebral sympathetic ganglia in prepubertal animals. Moreover, immunocytochemical studies have demonstrated an extranuclear localisation for the ERs in neurons of rat brain (Blaustein 1992).

It is important to emphasise that in the case of neurons associated with the ovary, the studies are scanty and basically linked to long-term effects on the physiology of the organ, when oestrogens are applied in early stages of the development (Shinohara *et al.* 1998, 2000, Lara *et al.* 2000, Koszykowska *et al.* 2011). The functions of ERs in sympathetic ganglia are related to the protection of the neurons against damage and the regulation of the growth of the neuronal prolongations (Sohrabji *et al.* 1994*a*, 1994*b*). Both ERs have similar affinities for binding E_2 (Mosselman *et al.* 1996, Kuiper *et al.* 1997) and could exert their effect through different signalling paths. However, the tissue distribution and level of expression of ERα and ERβ vary in sympathetic ganglia (Zoubina & Smith 2002).

It has been demonstrated that besides the well-studied transcriptional effects of E_2, there are rapid effects, i.e. occurring within seconds or minutes after the addition of E_2 (Song *et al.* 2005, Song & Santen 2006, Warner & Gustafsson 2006). These rapid effects are probably produced through non-genomic mechanisms, as has been observed in several tissues (Heldring *et al.* 2007). However, there is no agreement yet as to the participation of the classical oestrogenic receptors (Levin 2001, Deecher *et al.* 2003, Razandi *et al.* 2004) or whether there is a distinct membrane-associated receptor, such as GPR30 (Doolan & Harvey 2003, Dun *et al.* 2009).

It has been reported that oestrogen rapidly (between 5 and 15 min) increases the activity of mitogen-activated protein kinase, which persists for at least 2 h (Singh *et al.* 1999). In concordance with the above results, the addition of E_2 in the SMG produces a rapid increase in ovarian P_4 and A_2 release at 15 min, reaching a maximum at 60 min and a decrease at 120 min, in agreement with Singh *et al.* (1999). This effect observed on ovarian P_4 release might be due to the classical ERα found in the SMG, considering that by immunohistochemistry the ERβ was not observed in the ganglion, or else, to some other type of oestrogenic receptor, probably GPR30, which has been found at the sympathetic ganglionic level (Dun *et al.* 2009), might be involved in the rapid responses obtained on the release of ovarian P_4.

The effect of E_2 stimulus in the ganglion on ovarian P_4 and A_2 released might be accounted for by an increase in cAMP levels in neurons that constitute the SMG and the consequent increase in ovarian noradrenaline (NA) as

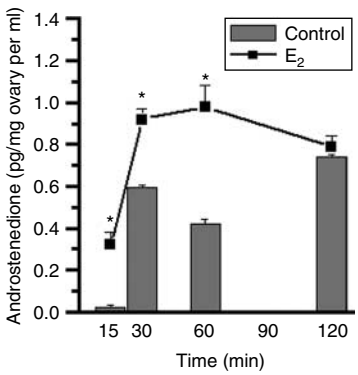

Figure 5 Effect of oestradiol (E_2) on ovarian androstenedione release in the superior mesenteric ganglion–ovarian nervous plexus–ovary system removed from rats on oestrus day. The systems were incubated in Krebs–Ringer buffer, plus ascorbic acid (0.1 mg/ml in Krebs–Ringer) solution, at 37 °C in an atmosphere of 95% O_2 to 5% CO_2 for 120 min without (control) and with the addition of E_2 (10^{-6} mol/l) to the ganglion compartment (experimental group). E_2, E_2 valerate. Each bar represents the mean±S.E.M. of six animals per experimental group. *$P<0.001$ compared with the control group (Student's *t*-test; ANOVA, Duncan's test).

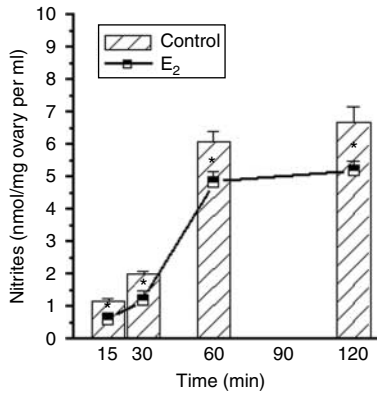

Figure 6 Effect of oestradiol (E_2) in the ganglion compartment on nitric oxide (NO) release in the incubation liquid of the ovarian compartment in the superior mesenteric ganglion–ovarian nervous plexus–ovary system of rats on oestrus day. The system was incubated in Krebs–Ringer buffer, plus ascorbic acid (0.1 mg/ml in Krebs–Ringer) solution, at 37 °C in an atmosphere of 95% O_2 to 5% CO_2 for 120 min without (control) and with the addition of E_2 (10^{-6} mol/l) to the ganglion compartments (experimental group). E_2, E_2 valerate. Values are the mean ± S.E.M. of six animals per experimental group. Columns with * indicate statistical significance of $^*P<0.001$ compared with the control group (Student's *t*-test; ANOVA, Duncan's test).

stated by Lara *et al.* (2002), with an increased capacity of the ovarian nerve terminals to incorporate and release NA. The effects of catecholamines in the ovary have been demonstrated in other experimental schemes (Aguado & Ojeda 1984, Barria *et al.* 1993).

Sosa *et al.* (2004) demonstrated in an *ex vivo* system that the cholinergic stimulus in the coeliac ganglion caused changes in NA and P_4 release at the ovarian level, according to the stage of the oestrous cycle, and this constitutes one of the factors governing the secretory activity of the ovarian steroids (Sosa *et al.* 2004). Recent studies using the SMG–ONP–ovary system have determined an increase in NA release in the ovarian compartment on oestrus day by cholinergic ganglionic stimulus (A Vega Orozco, C Daneri, D Bronzi, Z Sosa, AM Rastrilla, unpublished observations, 2010).

It has been demonstrated that the neuronal population in mesenteric ganglia is very heterogeneous, with numerous peptides and neurotransmitters. It has been shown in our research group that ganglionic stimulation in this *ex vivo* system releases not only catecholamines but also gonadotropin-releasing hormone (GnRH) and NO (A Vega Orozco, C Daneri, D Bronzi, Z Sosa, AM Rastrilla, unpublished observations, 2010). Garraza *et al.* (2004) demonstrated that the effect of other neurotransmitters such as substance P (SP) and neuropeptide Y (NPY) depends on the oestric cycle and that they can exert effects on the postganglionic terminal that reaches the ovary. These authors demonstrated that the addition of NA alone in hemi-ovaries incubations increases P_4 release while the addition of NPY, SP and vasoactive intestinal peptide (VIP) alone and together with NA generally decreases P_4 release at short times and increases it at long times. In addition, Miyamoto *et al.* (1993) reported that the addition of NPY, SP and VIP in cultures of luteal cells at short times and in the absence of luteinising hormone (LH) decreases the release of P_4, although using a different experimental scheme. In our system, which is free of humoral influences, there was an increase in P_4 by oestrogenic stimulation at ganglionic level.

With regard to the enzymes involved in the synthesis and degradation of P_4, the addition of E_2 in the ganglion increased the enzymatic activity and gene expression of 3β-HSD but not 20α-HSD in the ovary at 120 min of incubation in relation to the control. This result is surprising since there are no reports of enzymatic activity modulation at such short times. Variations in luteal cell cultures occurring at 36 h without neural influence and without the paracrine relations present in our *ex vivo* system have been reported (Tellería *et al.* 1995, Stocco & Deis 1996). It is important to note that although in this case E_2 in the ganglion has a short time effect (15 min), Singh *et al.* (1999) report that E_2 acts between 5 and 120 min. For this reason, the enzymatic activity and gene expression of ovarian 3β-HSD were measured at 120 min of incubation.

Considering that NA released at the ovarian level by the oestrogenic ganglionic stimulus might be responsible for the increase in ovarian P_4, the findings of this study are in agreement with postulations by Hsueh *et al.* (1984) and Miszkiel *et al.* (1999) that NA stimulates the activity of 3β-HSD. At the same time, P_4 produced by NA stimulus reduces the activity of monoaminooxidase (MAO) and catechol-*O*-methyl-transferase, the enzymes primarily responsible for the degradation of catecholamines (Mannisto & Kaakkola 1999, Eisenhofer *et al.* 2004). Thus, in this way, P_4 prolongs the half-life of NA and the duration of its stimulatory influence on P_4 synthesis.

Also, taking into account that Kotwica *et al.* (2004) and Rekawiecki *et al.* (2008) have postulated the autoregulation of P_4 synthesis in the corpus luteum, which affects the transcription of the genes encoding steroidogenic enzymes, it can be assumed that the values of P_4 can be due to the increase in the activity and gene expression of 3β-HSD caused by the same P_4 released throughout time. The accumulation of P_4 in ovarian steroidogenic cells is also influenced by the participation of the enzyme 20α-HSD, a P_4 degradation enzyme, which has been determined, cloned and sequenced in the corpus luteum of rats (Mao *et al.* 1997). In our case, this enzyme did not suffer variations at the studied times.

When the ERs antagonist was added, Txf alone in the ganglion inhibited ovarian P_4 release only at 60 min. Possibly, the effect of Txf occurs at the ganglionic level. In fact, Paech *et al.* (1997) and Kuiper *et al.* (1999) have demonstrated that the effect of Txf varies with the type of tissue and has action mechanisms that are different compared with traditional receptors (Paige *et al.* 1999, Zou *et al.* 1999). It is possible that, due to the E_2 level at this stage, the levels of the ERβ in the ganglion are too low

to be detected by immunohistochemistry. Both receptors are probably co-expressed at the ganglionic level, where ERβ antagonises the actions that depend on ERα as has been observed in other studies (Paech *et al*. 1997). Thus, the addition of an antagonist as Txf in the ganglion caused an inhibition of ovarian P_4 release at 60 min, an opposite to that of E_2 alone at the ganglionic level. The effects of E_2 and Txf alone were reverted by the addition of E_2 plus Txf, taking the P_4 levels to control values.

On the other hand, we postulate that the inhibitory effect of Txf alone in the ganglion might be through the ERβ. Webb *et al*. (1995) have demonstrated that anti-oestrogens such as Txf act as agonists of an alternative pathway (AP-1) to the classical ERs one, which is more potent in the presence of ERβ. Thus, it has been demonstrated that the ERβ potentially increases the transcription dependent on this alternative way in the presence of anti-oestrogens, but it is not activated in the presence of oestrogens, which might explain an inhibitory effect of Txf through this receptor that apparently acts as a repressor (Navarro Despaigne 2001). Thus, this effect is reverted by the addition of E_2 plus Txf at the ganglionic level. This issue will be the subject of future research.

It must be noted that in previous work, we demonstrated in the same SMG–ONP–ovary system that, besides catecholamines, other neurotransmitters such as NO may be released from the ONP by way of the ganglionic stimulus (Vega Orozco *et al*. 2010). Evidence has been reported that supports the involvement of NO in ovarian physiology (Fridén *et al*. 2000, Motta *et al*. 2001). In addition, it has been demonstrated that oestrogen up-regulates neuronal NOS expression through ERα in parasympathetic pelvic ganglion neurons (Berman *et al*. 1998). However, other investigators have shown that the various ERs on the cell surface are coupled to constitutive NOS-derived NO release (Stefano *et al*. 2003).

Our research group has demonstrated that the cholinergic agent in the coeliac ganglion stimulates NO release through NOS in prepubertal rat ovaries (Delgado *et al*. 2004). Studies with antagonists of the neuronal and inducible NO enzyme, both at the ganglionic and ovarian levels, showed that NO can reach the ovary by neural ways or be synthesised at the ovarian level (Delgado *et al*. 2004, Casais *et al*. 2006). In addition, Vega Orozco *et al*. (2006, 2010) have demonstrated that NO is involved in the formation and maintenance of the corpus luteum during the oestrous cycle through the ONP pathway.

Considering the above antecedents, it was decided to evaluate whether NO release is modified at the ovarian level by ganglionic oestrogenic stimulus in the *ex vivo* system and whether its presence is involved in ovarian steroidogenesis. Nitrites were found to decrease in the ovary compartment, which might be attributed to the direct action of oestrogen on the activity of one or more isoforms of the NO synthesis enzyme present in the ganglion, as was also observed by other authors (Stefano *et al*. 2003).

On the other hand, it is important to note that NO has been reported to decrease P_4 and A_2 release in *in vitro* cellular cultures (Olson *et al*. 1996, Dunnam *et al*. 1999, Fridén *et al*. 2000). Motta *et al*. (2001) have shown in rat that endogenous NO increased the production of glutathione and P_4 in corpora lutea in the middle stage of development. This result shows a dual, protective or pro-oxidising effect according to the stage of the oestrous cycle during which the tissue was obtained (Dong *et al*. 1999). Therefore, NO may be a luteolytic and/or luteotrophic factor on the corpus luteum, depending on the stage of the oestrous cycle (Olson *et al*. 1996, Fridén *et al*. 2000, Motta *et al*. 2001). In addition, Yamamoto *et al*. (1993) have demonstrated that NO might be involved in the modulation of the output and synthesis of NA, which is in agreement with the postulation that NA might be responsible for P_4 increase.

E_2 action in the ganglion observed in the present work probably favours the observed ovarian P_4 increase, having a trophic effect on the corpus luteum. On the basis of the results we obtained, we can propose that oestrogenic stimulus on the SMG would act in favour of the events that lead to the ovulation and later formation of the corpus luteum.

There is ample evidence suggesting that the sympathetic nervous system is involved in pathologies associated with the reproductive system. The role of oestrogen at the ganglionic level on ovarian steroidogenesis remains unclear and controversial. Anesetti *et al*. (2009) demonstrated that elevated levels of exogenous oestrogen during the prepubertal period in postganglionic sympathetic neurons increased sympathetic coeliac neuronal size and modified the expression of p^{75NTR} (p75 neurotrophin receptor), inducing a significant increase in catecholaminergic innervation of the ovary.

Polycystic ovary syndrome (PCOS) is associated with an abnormal activation of the sympathetic nervous system, showing an impaired metabolism of NA in adolescents suffering from this disease (Garcia-Rudaz *et al*. 1998).

The results of this study demonstrate that ERα activation in the SMG has an impact on ovarian steroidogenesis in rats at the ovarian level, thus providing evidence for the critical role of peripheral system neurons in the control of ovarian functions under normal and possibly under pathological conditions such as PCOS and ovarian cancer.

This work is expected to contribute new evidence for the clinical importance of these receptors in the therapy and prevention of ovarian pathology.

Materials and Methods

Animals

Virgin Holtzman strain adult female rats on oestrus (E) day weighing 250 g were used in all the experiments. The rats were kept in a light- (lights on from 0700 to 1900 h) and

temperature-controlled room (24±2 °C). Animals had free access to food (Cargill, SACI, Saladillo, Buenos Aires, Argentina) and tap water was available *ad libitum*. Vaginal smears were taken daily, and only the rats exhibiting at least two 4-day consecutive oestrous cycles were used. Groups of six animals were used for each group utilised in the experimental procedure. The experiments were performed per duplicate according to the procedures approved in the 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 (number protocol: B17/04, ordinance CD 006/02).

Chemicals

The compounds E_2 valerate (E_2), Txf, ascorbic acid, BSA fraction V, sulphanilamide and N-(1-naphthyl)ethylenediamine were purchased from Sigma-Aldrich Co. Other reagents and chemicals were of analytical grade. 1,2,6,7-[^{3}H]-P_4 (107.0 Ci/mmol), 1,2,6,7-[^{3}H]-androst 4-ene-3,17 dione (115.0 Ci/mmol) and 17-β-2,4,6,7-[^{3}H]-estradiol (102.0 Ci/mmol) were provided by New England Nuclear Products (Boston, MA, USA).

Experimental procedures

Extraction of SMG fixed in situ for localisation of oestrogenic receptors by immunohistochemistry

Rats on oestrus day were anaesthetised under ether bell. The SMG and nearby ganglia were fixed *in situ* with Bouin solution for subsequent extraction and application of immunohistochemistry.

Extraction of the SMG–ONP–ovary system

Groups of six animals on oestrus day were used. The procedure was carried out between 1500 and 1600 h taking into account previous descriptions of the anatomical trajectory of this neural pathway as a guide (Lawrence & Burden 1980, Klein & Burden 1988*a*, 1988*b*, Vega Orozco *et al*. 2006).

The piece of tissue utilised in this work corresponds to the whole SMG where the principal neurons are located, whose axons constitute the ONP that reaches the ovary, without the presence of the coeliac ganglion (Vega Orozco *et al*. 2006).

The anatomical localisation of the SMG and coeliac ganglia (CG) is different, as well as the neuron type and size. The SMG was extracted from the area proximal to the CG, proximal to the renal artery and abdominal aorta artery. It is important to clarify immediately before and after incubation (120 min) that the presence of the SMG was confirmed in the system by the application of routine histological techniques. The presence of the ganglion was verified in the removed system before incubation in similar conditions to the systems that were going to be used later.

Rats were anaesthetised with ether under a bell, and the *ex vivo* system was immediately removed by dissection.

Each system consisted of the ovary, the fibres constituting the ONP, parallel to the ovarian artery, and the SMG, surrounded by some small ganglia.

The total surgical procedure was completed in 1–2 min. The strip of tissue was carefully dissected, avoiding contact between the surgical instruments, the nerve fibres and the ganglion to prevent spontaneous depolarisations of the nerves, which might have been caused by inappropriate contact.

The extracted systems were washed with incubation solution and immediately placed in a cuvette with two compartments. Each cuvette contained 2 ml of work solution, Krebs–Ringer bicarbonate buffer, pH 7.4, in the presence of dextrose (0.1 mg/ml) and BSA (0.1 mg/ml) as described in previous studies (Sosa *et al*. 2000, Vega Orozco *et al*. 2006).

The ganglion was placed in one compartment and the ovary in the other, both joined by the ONP, which was maintained humid with the work solution. The system was immediately preincubated in a metabolic bath at 37 °C in a saturated O_2/CO_2 (95–5%) atmosphere for 15 min to achieve the stabilisation of the system as described in a previous study (Vega Orozco *et al*. 2006).

After 15 min of preincubation (time 0 of incubation), the Krebs–Ringer solution was changed in both compartments, and ascorbic acid (0.1 mg/ml in Krebs–Ringer) was added as an antioxidant agent (Koh & Hille 1997) in the ganglion compartment whereas the Krebs–Ringer solution was added in the ovary compartment. The P_4, A_2 and nitrite values released under these conditions were considered as control values (control group).

The experimental groups proposed were a) SMG–ONP–ovary system with the addition of E_2 (10^{-6} mol/l) in the ganglion compartment, b) SMG–ONP–ovary system with addition of Txf (10^{-6} mol/l) in the ganglion compartment and c) SMG–ONP–ovary system with addition of Txf (10^{-6} mol/l) and E_2 (10^{-6} mol/l) 5 min later in the ganglion compartment. Groups b and c were only used for P_4 release determination and for evaluating whether Txf acts as an oestrogenic antagonist at ganglionic level. The control group was SMG–ONP–ovary system without treatment (control). The different substances were dissolved in equal concentration (10^{-6} mol/l) and volumes (2 ml) of Krebs–Ringer buffer plus ascorbic acid.

For the system standardisation, the dose/response curve was obtained for this *ex vivo* system and at the working times used. The curve shows the highest P_4 increase with the used dose (10^{-6} mol/l) compared with other doses used.

Liquid samples from the ovary compartment (250 µl) were collected at 15, 30, 60 and 120 min and kept at −20 °C until the determination of P_4 and A_2 by RIA and NO using the Griess method.

The results were expressed as the nanogram of P_4 and picogram of A_2 per milligram of ovarian tissue per millilitre and nitrites as nanomol of nitrites per milligram of ovarian tissue per millilitre (nmol/mg ovary/ml) all against the incubation time. The respective corrections were made in all cases considering the volume extracted in each tested period. When the system incubation was finished (120 min), the ovaries were kept at −80 °C until determination of 3β-HSD and 20α-HSD activities and gene expression.

Immunohistochemistry

After the experimental treatment, the SMGs were fixed in 4% paraformaldehyde, included in paraffin and sectioned at 5 µm.

For immunohistochemistry, sections were deparaffinised, rehydrated and rinsed in distilled water. After antigen retrieval using microwave treatment at 160 W in 10 mM sodium citrate, pH 6.0, for 30 min, sections were washed in PBS and incubated for 20 min in 0.3% hydrogen peroxide in PBS to inhibit endogenous peroxidases. Then, sections were permeabilised by 30-min incubation in PBS containing 0.5% Triton X-100 and blocked in PBS containing 0.5% Triton X-100 and 2% BSA for 1 h. Sections were incubated overnight at 4 °C with the appropriate primary antibody diluted in blocking solution. The primary antibodies used were as follows: a polyclonal rabbit anti-ERα (ERα, MC-20; SC-542, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:400) and a rabbit polyclonal anti-serum against ERβ (ERβ, H-150; SC-8974 Santa Cruz Biotechnology; 1:100). Subsequently, sections were rinsed in PBS, incubated with biotinylated secondary antibody (1:300 in blocking solution) for 1 h, washed in PBS and incubated for 1 h with avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA). After several washes in PBS, sections were exposed to a 0.02% 3,3′-diaminobenzidine (Sigma-Aldrich Co.) solution. The reaction was stopped in tap water and some sections were counterstained in haematoxylin (20 s), dehydrated in ethanol, cleared in xylene, coverslipped and examined on a Nikon photomicroscope. Each immunoreaction was performed on at least three slides from each set of ganglia. Sections of uterus and ovary were processed in parallel under identical conditions and used as positive controls; staining in these tissues showed strong, predominantly nuclear localisation with both antibodies. To confirm the specificity of the staining, selected sections were incubated without primary or secondary antibodies with positive and negative controls being included.

Nitrite assay

Levels of nitrite, a water-soluble metabolite of NO, were measured spectrophotometrically by the Griess method and were expressed in nanomole of nitrite per milligram of ovarian tissue per millilitre (nmol/mg ovary per ml; Egami & Taniguchi 1974). The samples (50 µl) were immediately mixed with Griess reagent (sulphanilamide with N-(1-naphthyl) ethylenediamine/HCl). After 10 min of incubation at room temperature, it was read for absorbance at 540 nm, and nanomoles of nitrite were determined using a standard curve. The assay sensitivity was <2.5 nmol/ml. The intra-assay coefficient of variation for all the assays was $<10.0\%$.

P_4 and A_2 assay

Steroids were measured in duplicate by RIA. The anti-sera to P_4 were kindly provided by Dr R Deis (Laboratorio de Reproducción y Lactancia, Mendoza, Argentina). P_4 was measured in nanogram per milligram ovary per millilitre and assay sensitivity was <5 ng P_4/ml. A_2 was expressed as picogram per milligram of ovarian tissue per millilitre (pg/ml ovary per ml), all against incubation time on the systems. The assay sensitivity was <10 pg A_2/ml. The inter- and intra-assay coefficients of variation in all the assays were $<10.0\%$. The result of each experiment is expressed with respect to the corresponding control group.

Enzymatic activity

The activities of 3β-HSD and 20α-HSD were measured according to Kawano *et al.* (1988) with a slight modification. The ovary from each animal was homogenised in 0.7 ml of 0.1 M Tris–HCl, 1 mM EDTA (pH 8) at 0 °C with a glass homogeniser. The homogenates were centrifuged at 105 000 ***g*** for 60 min. The supernatant fluids were used for the assay of 20α-HSD activity. The precipitates were rehomogenised with 0.7 ml of 0.25 M sucrose and centrifuged at 800 ***g*** for 5 min. The supernatants were used as the enzyme solution for the assay of 3β-HSD activity. The substrates for 3β-HSD and 20α-HSD were pregnenolone (5 µg) and 20α-hydroxypregn-4-en-3-one (12.5 µg) respectively. Both enzyme activities were assayed spectrophotometrically, depending on the increase in NADH or NADPH in 1 min at 37 °C, and the values were expressed as mU/mg protein/min. The method of Lowry *et al.* (1951) was used for protein determination with BSA as the standard.

RNA isolation and RT-PCR analysis

Once the ovaries were defrosted, the total RNA was extracted using the TRIzol-reagent method (Invitrogen Life Technologies), following the manufacturer's instructions for the RNA extraction (Chomczynski 1993).

Two micrograms of total RNA were reverse transcribed using a Moloney murine leukaemia virus (MMLV) RT and random primers. First, a prereverse transcription (pre-RT) was carried out, using random primers, by incubating for 10 min at room temperature and then 45 min at 42 °C. The buffer RT, the enzyme MMLV-RT (Invitrogen Life Technologies) and a mixture of dNTPs were added to the total pre-RT product, and it was carried to a final volume of 50 µl. The mixture was incubated for 60 min at 42 °C, then 15 min at 70 °C and finally the reverse transcriptase activity was inactivated by incubating the tubes in an ice bath.

For amplification of the RT products, the reaction mixture consisted of 10 µl Go Taq Green reaction buffer, 0.2 mM dNTPs, 0.5 µM specific oligonucleotide primers and 1.25 U Go Taq DNA polymerase (Promega, Inc.) in a final volume of 50 µl. Amplification was carried out for 30 cycles using 93 °C for denaturation (1 min), 59 °C for annealing (1 min) and 72 °C for extension (15 min) in an Eppendorf thermal cycler.

Specific primers for 3β-HSD were 5′-CTGCCTGGTGACAGGAGCAGG-3′ and 5′-GCCAGCACTGCCTTCTCGGCC-3′ and for 20α-HSD were 5′-TTCGAGCAGAACTCATGGCTA-3′ and 5′-CAACCAGGTAGAATGCCATCT-3′. Each reaction also included primers (5′-CAAGACTGAGTGGCTGGATGG-3′ and 5′-ACTTGAAGGGGAATGAGGAAAA-3′) to amplify protein cyclophilin A as an internal control. The predicted sizes of the PCR-amplified products were 489 bp for 3β-HSD, 440 pb for 20α-HSD and 293 pb for cyclophilin A. Reaction products were electrophoresed on 1.5% agarose gels, visualised with ethidium bromide (5.5 mg/ml) and examined by u.v. transillumination. Band intensities of RT-PCR products were quantified using ImageJ (NIH, Bethesda, MA, USA; http://rsb.info.nih.gov/ij/) and expressed as arbitrary units.

Statistical analysis

All data are presented as the mean ± S.E.M. in each group of six rats. Differences between two groups were analysed with the Student's *t*-test. ANOVA I followed by the Tukey's test was used for between-group comparison. A value of $P<0.05$ was considered statistically significant (Snedecor & Cochram 1976).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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