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Involvement of the coeliac ganglion in the luteotrophic effect of androstenedione in late pregnant rats

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Abstract

Using the *ex vivo* coeliac ganglion-superior ovarian nerve-ovary system at the end of pregnancy when luteal regression starts, we investigated whether, when administered systemically or when added directly to the ganglion compartment, androstenedione (A_2) can reverse such regression, and whether the neural (noradrenaline (NA)) and endocrine (A_2) joint action modifies the release of ovarian progesterone. The experimental groups were as follows: group 1 – A_2 injected systemically 48 h before incubation of the system $(A_2)_s$; group 2 – A_2 directly added to the ganglion compartment $(A_2)_g$; group 3 – A_2 injected 48 h before incubation of the system with NA in the ganglion compartment (A_2 + NA); group 4 – A_2 plus NA added to the ganglion compartment $(NA + A_2)_g$. The controls were *ex vivo* systems without treatment (control), and with the addition of NA alone in the ganglion compartment (NA). The results were as follows. For $(A_2)_s$ versus control, progesterone increased on days 19 and 21 of pregnancy at all the studied times and only at 180 min on day 20. For (A_2 + NA) versus $(A_2)_s$, progesterone increased on days 19 and 21. For (A_2 + NA) versus NA, progesterone increased at all the studied times on days 19 and 21 and at 180 min on day 20. For $(A_2)_g$ versus control, progesterone significantly increased every pregnancy day. For $(NA + A_2)_g$ versus $(A_2)_g$, progesterone decreased at 120 and 180 min on day 19. For $(NA + A_2)_g$ versus NA, progesterone increased on days 20 and 21. We can conclude that A_2 can reverse the functional regression of the corpus luteum either systemically or, what is more surprising, when directly added to the coeliac ganglion, whose action on the ovary is exerted via superior ovarian nerve.
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Introduction

The coeliac ganglion, part of the sympathetic prevertebral chain, is constituted mainly by principal neurons and interneurons such as the peptidergic and the intensely fluorescent (SIF) (Eränkö 1978, Abe *et al.* 1983, Matthews 1989, Prud'Homme *et al.* 1999). The sympathetic ganglia possess a great variety of specific receptors and neurotransmitters, among them catecholamines (Klein & Burden 1988), neuropeptides (Dalsgaard *et al.* 1983) and gaseous neurotransmitters such as nitric oxide (Morales *et al.* 1995). It is interesting to point out the presence of a profuse capillary plexus that constitutes a microcirculation among the different ganglionic structures (Chau & Lu 1995).

Previous studies have shown that the humoral environment influences the functioning of the coeliac ganglion, thus affecting the ovary via the superior ovarian nerve (Aguado 2002).

The superior ovarian nerve is mostly constituted by adrenergic fibres whose origin neurons are mainly located in the coeliac ganglion (Baljet & Drukker 1979) and whose terminals act directly upon the secretory part of the ovary, including the theca externa and interna of the follicle, the corpus luteum capsule and the interstitial gland. However, direct contact of nerves with luteal and granulosa cells has not been shown (Lawrence & Burden 1980, Erickson *et al.* 1985, Klein & Burden 1988). The ovarian noradrenergic stimulation affects progesterone secretion and its synthesis by an increase of cytochrome P450scc and 3β-HSD enzymatic activity. In corpus luteum, this effect is mediated via β_1 and β_2 receptors (Pesta *et al.* 1994, Miszkiel & Kotwica 2001) with activation of cAMP (Lefkowitz, 1987). Besides acting on the luteal β-receptors, noradrenaline (NA) acts on ovarian vascular adrenoreceptors (Reynolds & Ford 1984) and adipose cell β-receptors. The stimulation of lipolysis and the increased blood flow through the ovary could supply the corpus luteum with serum-derived lipoproteins as a source of cholesterol for luteal steroidogenesis (Williams 1989). In studies in conscious heifers, the infusion of NA into the abdominalis aorta stimulated the secretion of progesterone within a few minutes in a dose-dependent manner (Kotwica *et al.* 2002). De Bortoli *et al.* (1998,

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2000) demonstrated that a central adrenergic stimulus produces acute effects on the release of steroids from the ovary. Ovarian progesterone changes could be attributed to signals coming from ganglionic neurons, which are affected by the central adrenergic stimulation. By use of the *ex vivo* coeliac ganglion-superior ovarian nerve-ovary system (coeliac ganglion-SON-ovary) of rats in the second half of pregnancy, the release of ovarian progesterone was modified at 30 min of incubation with NA in the ganglion compartment (Casais *et al.* 2001). This rapid ovary response was probably the result not only of a direct influence of NA on the luteal cells via β-receptors, but also of other factors released via SON which could amplify this effect indirectly.

It is already known that progesterone is a survival factor in the corpora lutea (Sugino *et al.* 1997, Tellería *et al.* 1999, Villavicencio *et al.* 2002). At the end of pregnancy, the corpora lutea suffer a regression process that decreases their capacity to produce progesterone (functional regression) and causes the subsequent involution of the luteal tissue, including steroidogenic and nonsteroidogenic cells (structural regression) (Bowen-Shauver & Tellería 2003).

On the other hand, androstenedione (A_2), the principal androgen in rat, has a well-known antiapoptotic affect either through its intraluteal conversion to oestradiol (Gibori *et al.* 1988) or through a non-genomic mechanism (Carrizo *et al.* 1994, Thordarson *et al.* 1997, Machelón *et al.* 1998). It has also been suggested that A_2 could have a dual effect: a non-genomic effect that would lead to the activation of a complex cascade of signal transduction (Cato & Peterziel 1998) and a subsequent genomic event.

With these antecedents, we thought it interesting to investigate whether A_2 is capable of reversing the luteal functional regression, whether systemically administered (by injection 48 h before the system incubation) or directly added to the ganglion compartment.

Considering that NA action in the ganglion compartment decreases as pregnancy progresses (Casais *et al.* 2001) and that A_2 inhibits the luteal functional regression, we also decided to analyse the neural and endocrine joint action of NA and A_2 on the release of ovarian progesterone at the end of rat pregnancy.

Materials and Methods

Animals

Adult female rats bred in our laboratory (originally Holtzman albino strain) aged 2–3 months and weighing 220 ± 50 g were used. The rats were housed under controlled light (lights on from 0700 to 1900 h) and temperature (24 ± 2 °C) conditions, and allowed *ad libitum* access to water and food (rat chow-Cargil SACI; Saladillo, Buenos Aires, Argentina).

To induce pregnancy, female rats were caged individually with fertile males beginning on the afternoon of prooestrus. Positive mating was verified on the following morning by identifying sperm or copulation plugs in the vagina. This day was designated as day 0 of pregnancy. In our laboratory, rats usually give birth on day 22.

Animals were handled according to the procedures approved in the UFAW *Handbook on the Care and Management of Laboratory Animals*, vol. 1: *Terrestrial Vertebrates* (7th edn), edited by T Poole (1999), and the *Guide for Animal Use and Handling* of the National University of San Luis.

Systemic A_2

A_2 was systematically administered 48 h before the incubation of coeliac ganglion-SON-ovary system with and without addition of NA in the ganglion compartment.

Drugs

Drugs used were:

- hydrochloride of L-D-NA (Sigma)
- 4-androsteno-3,17,diona (A_2) (Sigma).

A_2 preparation for injection

A_2 was dissolved in benzilic alcohol (2–5% of the final volume) to a sufficient concentration to inject each animal with 10 mg A_2/0.2 ml vegetable oil. The administration to animals was subcutaneous (s.c.).

Procedure

Groups of six animals each on days 17–19 of pregnancy were used for each experimental procedure with the following distribution:

- group 1: pregnant rats on day 17
- group 2: pregnant rats on day 18
- group 3: pregnant rats on day 19.

Rats were administered (s.c.) 10 mg A_2 /0.2 ml oil vehicle. This procedure was carried out at 0900 h on the pregnancy days mentioned (days 17–19). The animals were caged individually, and, at 0900 h on days 19–21 of pregnancy, that is to say, 48 h after injecting A_2, they were anaesthetized under ether bell, and the coeliac ganglion-SON-ovary system was immediately extracted (Casais *et al.* 2001).

The fetuses were removed and killed under ether anaesthesia. Briefly, the coeliac ganglion-SON-ovary system was placed in a cuvette with two compartments, each containing 2 ml Krebs-Ringer bicarbonate buffer, pH 7.4; 0.1 mg glucose/ml; and 0.1 mg albumin/ml. After 30-min preincubation, the liquid was removed from the cuvette, buffer was added to the ovarian compartment, and buffer plus 0.1 mg ascorbic acid/ml was added to the ganglion compartment. The previous description corresponds to the group $(A_2)_s$. Previous injection of A_2 plus 10^{-6} mol NA/l in the ganglion compartment constituted the group (A_2 + NA).

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Periodic extractions of incubation liquid were made from the ovary (250 μl) at 30, 60, 120 and 180 min, and they were stored in a freezer at −20 °C until the determination of progesterone by RIA. The corresponding corrections were made in all cases, taking into consideration the volume extracted in each tested period.

A_2 in coeliac ganglion

The coeliac ganglion-SON-ovary system was incubated in the presence of A_2 with or without the addition of NA to the ganglion compartment.

10^{-6} mol A_2/l preparation

A_2 was dissolved in benzilic alcohol (2–5% of the final volume), and a solution at final concentration of 10^{-6} mol A_2/l in Krebs-Ringer bicarbonate buffer, pH 7.4; 0.1 mg glucose/ml; and 0.1 mg albumin/ml was added directly to the ganglion compartment. This solution was used for group $(A_2)_g$.

The solution $(NA + A_2)_g$ was prepared in such a way that the joint preparation of NA and A_2 constituted a solution with final concentration of 10^{-6} mol NA + A_2/l in Krebs-Ringer bicarbonate buffer, pH 7.4; 0.1 mg glucose/ml; and 0.1 mg albumin/ml. This solution was directly added to the ganglion compartment.

Procedure

Rats on days 19–21 of pregnancy were anaesthetized under ether bell, and the coeliac ganglion-SON-ovary system was extracted by the procedure previously described. The addition of 10^{-6} mol A_2/l plus 0.1 mg ascorbic acid/ml to the ganglion compartment characterized group $(A_2)_g$. Group $(NA + A_2)_g$ had 10^{-6} mol NA + A_2/l plus 0.1 mg ascorbic acid/ml in the ganglion compartment. Progesterone determination in the ovarian incubation liquid was measured by RIA at all the studied times.

Progesterone assay

Progesterone was measured by RIA using antiserum raised against progesterone-11-BSA conjugate in rabbits, provided by Dr R P Deis (Laboratorio de Reproducción y Lactancia Mendoza, Argentina). The sensitivity, variability and cross-reaction of this RIA have been reported previously (Bussmann & Deis 1979). The sensitivity of the assay is less than 5 ng progesterone/ml serum, and the inter- and intra-assay coefficients of variation were less than 10%. This assay has been previously validated (Bussmann & Deis 1979).

Statistical analysis

Results are presented as mean ± S.E.M. in each group of six rats. Differences between two groups were analysed with Student's *t*-test. The analysis of the variance (ANOVA I), followed by Duncan's multiple-range test was used for several comparisons. A value of $P < 0.05$ was considered statistically significant (Snedecor & Cochran 1976).

Results

Systemic effect of A_2 on the release of ovarian progesterone in the coeliac ganglion-SON-ovary system

Figure 1A–C shows that in the coeliac ganglion-SON-ovary system extracted from animals on days 19 and 21 of pregnancy, with previous s.c. administration (48 h) of 10 mg A_2/0.2 ml oil vehicle $(A_2)_s$, the levels of progesterone increased significantly when compared with the control values at all the studied times ($*P < 0.05$). On day 20, a decrease at 30 and 60 min (0.06 ± 0.008 compared with 0.13 ± 0.02 ng/mg ovary, $*P < 0.05$; 0.08 ± 0.018 compared with 0.17 ± 0.03 ng/mg ovary, $*P < 0.05$ respectively) and an increase at 180 min (0.26 ± 0.02 compared with 0.18 ± 0.03 ng/mg ovary, $*P < 0.05$) were observed in relation to control values. The comparison was done with the control rats without treatment since the s.c. administration of 0.2 ml oil vehicle alone did not modify the release of progesterone (data not shown).

When we added 10^{-6} mol NA/l to the ganglion compartment (A_2 + NA) besides injecting 10 mg A_2/0.2 ml oil vehicle, 48 h before the incubation of the coeliac ganglion-SON-ovary system, the release of progesterone on day 19 increased significantly in relation to $(A_2)_s$ at all the studied times ($\bullet P < 0.05$). On day 20, a decrease of progesterone was observed only at 120 min (0.09 ± 0.03 compared with 0.17 ± 0.03 ng/mg ovary, $\bullet P < 0.05$), and on day 21, there was a significant increase of progesterone at 60 and 180 min (0.21 ± 0.03 compared with 0.09 ± 0.016 ng/mg ovary, $\bullet P < 0.01$; 0.24 ± 0.04 compared with 0.13 ± 0.01 ng/mg ovary, $\bullet P < 0.01$ respectively).

When we compared the experimental groups (A_2 + NA) in relation to the stimulation of coeliac ganglion with 10^{-6} mol NA/l, it was observed that the neural and endocrine joint action increased the release of progesterone on days 19 and 21 at all the studied times ($\#P < 0.05$), and on day 20 only at 180 min (0.24 ± 0.03 compared with 0.10 ± 0.005 ng/mg ovary, $\#P < 0.01$).

Ganglionic effect of A_2 in the coeliac ganglion-SON-ovary system on the release of ovarian progesterone

Figure 2A–C shows that the presence of 10^{-6} mol A_2/l in coeliac ganglion $(A_2)_g$ increased the release of progesterone in relation to the control at all the studied times on the days of pregnancy studied (days 19–21) ($*P < 0.01$).

The analysis of NA and A_2 joint action in the coeliac ganglion $(NA + A_2)_g$ shows that the release of ovarian progesterone tends to decrease in relation to the sole action of $(A_2)_g$. On day 19 of pregnancy, the

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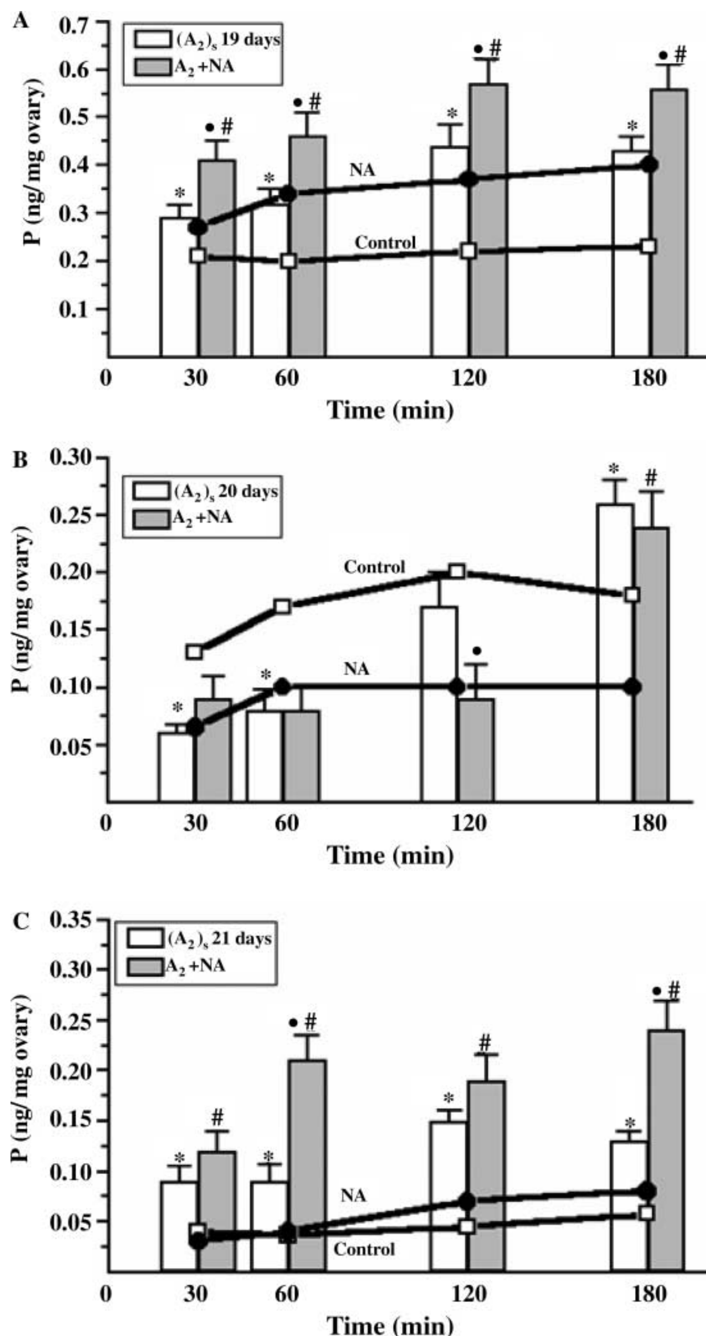

Figure 1 Effect of A_2 injection 48 h before, and subsequent incubation of the coeliac ganglion-superior ovarian nerve-ovary system with NA in the ganglion compartment, on the release of ovarian progesterone. The work was carried out with rats on days 19 (A), 20 (B) and 21 (C) of pregnancy. $(A_2)_s$ (A_2 injection, 10 mg A_2/0.2 ml by rat, s.c., 48 h before and incubation of the coeliac ganglion-SON-ovary system with Krebs-Ringer bicarbonate buffer, plus 0.1 mg ascorbic acid/ml, at 37 °C in an atmosphere of 95% O_2-5% CO_2 for 180 min). $A_2 + NA$ (A_2 injection, 10 mg A_2/0.2 ml by rat, s.c., 48 h and incubation of the coeliac ganglion-SON-ovary system with 10^{-6} mol NA/1 in the ganglion compartment. Control (incubation with buffer alone of the coeliac ganglion-superior ovarian nerve-ovary system obtained from animals without previous A_2 injection). NA (incubation of the coeliac ganglion-superior ovarian nerve-ovary system obtained from animals without previous A_2 injection and with 10^{-6} mol NA/1 in the ganglion compartment). Control and NA (Casais *et al.* 2001). Results are expressed as mean ± S.E.M. of six animals per experimental group (ANOVA I and Duncan test of multiple range) $P < 0.05$ and $P < 0.01$. $(A_2)_s$ versus control (*); $(A_2)_s$ versus $A_2 + NA$ (•); $A_2 + NA$ versus NA (#).

inhibition of the release of progesterone was significant at 120 and 180 min (120 min: 0.32 ± 0.05 compared with 0.58 ± 0.06 ng/mg ovary, •$P < 0.01$; 180 min: 0.30 ± 0.03 compared with 0.55 ± 0.05 ng/mg ovary, •$P < 0.01$); on day 20, at 60 min (0.29 ± 0.03 compared with 0.40 ± 0.03 ng/mg ovary, •$P < 0.05$); and on day 21, at 120 min (0.15 ± 0.015 compared with 0.21 ± 0.016 ng/mg ovary, •$P < 0.05$).

Finally, when comparing the experimental groups $(NA + A_2)_g$ in relation to the stimulation of coeliac ganglion with 10^{-6} mol NA/l, we observed that, through the neural and endocrine joint action, the release of progesterone did not exhibit significant changes on day 19, whereas on days 20 and 21, it increased significantly at all the studied times (#$P < 0.01$).

Discussion

As stated in the introduction, this work demonstrates that A_2 might act through an endocrine pathway and, surprisingly, through a neural pathway, since it reverses the functional regression of the corpus luteum either systemically

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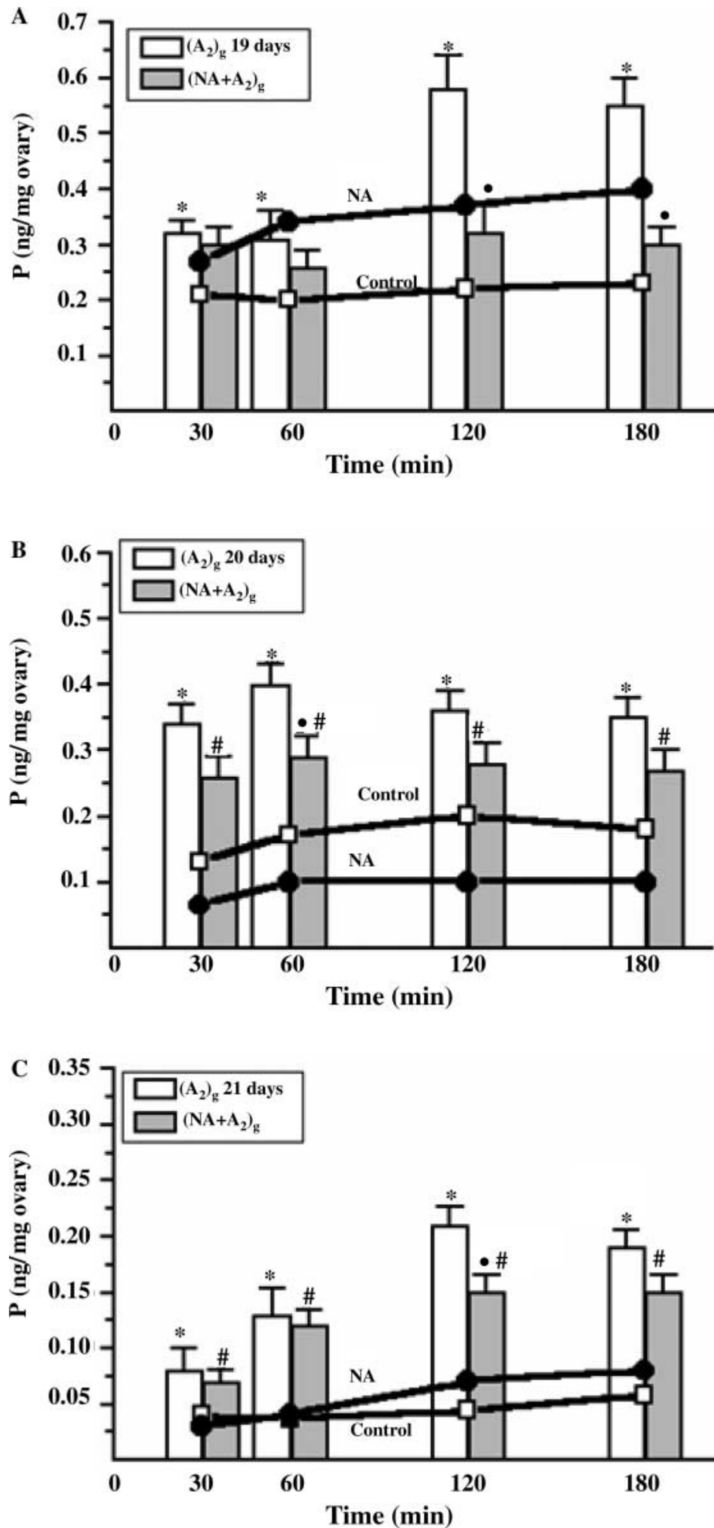

Figure 2 Effect of the incubation of the coeliac ganglion-superior ovarian nerve-ovary system in the presence of NA + A_2 in the ganglion compartment on the release of ovarian progesterone. The work was carried out with rats on days 19 (A), 20 (B) and 21 (C) of pregnancy. When the coeliac ganglion-SON-ovary system is incubated with 10^{-6} mol A_2/l in the coeliac ganglion, the group is $(A_2)_g$. When A_2 and NA, in 10^{-6} mol NA + A_2/l are added together on the coeliac ganglion, the group is considered $(NA + A_2)_g$. Control (incubation of the coeliac ganglion-superior ovarian nerve-ovary system with buffer). NA (incubation of the coeliac ganglion-superior ovarian nerve-ovary system, with 10^{-6} mol NA/1 alone in the ganglion compartment). Control and NA (Casais *et al.* 2001). Results are expressed as mean ± S.E.M. of six animals per experimental group (ANOVA I and Duncan test of multiple range) $P < 0.05$ and $P < 0.01$. $(A_2)_g$ versus control (*); $(A_2)_g$ versus $(NA + A_2)_g$ (•); $(NA + A_2)_g$ versus NA (#).

or when added to the ganglion compartment. On the other hand, the NA and A_2 joint action is greater when A_2 is administered 48 h before incubation. Its luteotrophic effect is likely to permit the rescue of the corpora lutea from regression. Consequently, NA action in the ganglion might be more effective.

Although A_2 is a weak androgen, it is the androgen of highest concentration in the circulation in rat (Gibori *et al.* 1988). Previous research has demonstrated that A_2 stimulates the luteal production of progesterone in rodents (Carrizo *et al.* 1994, Tellería *et al.* 1995, Thordarson *et al.* 1997). It has also been demonstrated that A_2 interferes with

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luteal regression by inhibiting apoptosis and stimulating progesterone production (Goyeneche *et al.* 2002).

A_2 acts at two distinct levels: through the nuclear receptor expression (Goyeneche *et al.* 2002) and through a non-genomic mechanism, as it occurs in the human luteinized granulosa cells, in which this androgen specifically modifies the intracellular levels of calcium. This effect could not be either imitated with testosterone or blocked by androgen receptor blockers (Machelón *et al.* 1998).

When working with the coeliac ganglion-SON-ovary system of late-pregnancy rats with and without previous A_2 injection 48 h before (systemic A_2 effect), we observed that the presence of A_2 in circulation increased the release of progesterone in the ovarian compartment, leading us to conclude that A_2 shows trophic effect. This agrees with the previously mentioned findings.

Besides, as the coeliac ganglion-SON-ovary system offers the ability to analyse neuroendocrine effects (Sosa *et al.* 2000, 2004, Casais *et al.* 2001, Delgado *et al.* 2004), we incubated *ex vivo* coeliac ganglion-SON-ovary systems extracted from animals with previous A_2 injection in the presence of NA in the ganglion compartment, and we determined the levels of ovarian progesterone release. The presence of catecholamine in the coeliac ganglion increased ovarian progesterone not only in relation to A_2 injection but also when NA was added to the coeliac ganglion without previous A_2 injection, leading us to suggest that A_2 may prevent regression of the corpus luteum.

These results agree with our previous work (Casais *et al.* 2001) in which we demonstrated that the adrenergic agents in the coeliac ganglion assist gestation physiology; that is, they decrease the release of ovarian progesterone at the end of pregnancy. However, when A_2 is present in the circulation, NA in the coeliac ganglion has an effect in late pregnancy (especially on day 21), indicating that androgen has kept the biosynthetic structure of progesterone at ovarian level. Yet, under these experimental conditions, it is not possible to determine whether A_2 exerts its effects at ovarian or neural level.

There is an extensive literature about the action of gonadal steroids on the central nervous system (McEwen 1981, McEwen & Alves 1999, Woolley 1999, Hammond *et al.* 2001). However, few studies have been devoted to the action of gonadal steroids on the peripheral nervous system. Gejman and Cardinali (1983) suggest that muscarinic cholinergic neurotransmission in bovine and rat superior cervical ganglia may be affected by hormones.

In their review, Papka and Mowa (2003) show the presence of oestrogen receptors in spinal cord neurons and sensory and autonomic ganglionic neurons in sites that are associated with innervation of the female reproductive organs, and explain the role that oestrogen may play in these neurons and their circuits. On the other hand, Chen and Hua (1987) have shown the fast effect of glucocorticoid on neuronal membrane of hippocampal slices of rat and isolated coeliac ganglion of guinea pig. Hua and Chen (1989) also suggest that glucocorticoid can act non-genomically through its neuronal membrane receptor.

It is also known that the androgens can increase the volume, neuron number and synapses of developing rat superior cervical ganglion (Wright *et al.* 1991). Moreover, Félix *et al.* (2001) have shown an effect of testosterone on the electrical properties and nicotinic transmission of the major pelvic and coeliac ganglion neurons (nongenomic effect). The steroids are known to act through the steroid receptor and activate genomic pathways. Alternatives to the classical theory are theories of the non-genomic mechanisms, of which the hypothesis of membrane receptor-mediated effects in neurons is very important since rapid membrane effects form the basis of neuronal function.

Considering that a profuse fenestrate capilaris plexus has been described in the structure of prevertebral ganglia (Chau & Lu 1995), it is reasonable to suggest that A_2 injected systemically reaches the ganglionic cells through the plexus. Aguado (2002) demonstrates that addition of serum to ganglion compartment in the coeliac ganglion-SON-ovary system increases the release of progesterone. These *in vitro* results support the idea that, *in vivo*, the humoral environment is highly influential on the functioning of the coeliac ganglion, thus affecting the ovary by a neural pathway.

When the direct ganglionic effect of A_2 was analysed in the coeliac ganglion-SON-ovary system, the results were highly surprising. The levels of progesterone obtained by the application of A_2 to the coeliac ganglion in relation to the controls were significantly higher on all the analysed days, and on day 21 they took longer (120–180 min) to reach the control levels of day 19. This would clearly demonstrate the neural action of the androgen in preventing the functional regression of the corpus luteum. We also compared the effect of androgen on the coeliac ganglion in relation to the addition of NA to the ganglion compartment (Casais *et al.* 2001), and we observed that the levels of release of progesterone with A_2 in the coeliac ganglion were higher than those obtained with the addition of NA to the ganglion compartment on all the pregnancy days analysed. This action is more effective as the pregnancy age progresses. These facts support the idea that the steroid in the coeliac ganglion exerts a strong luteotrophic effect differently from the catecholamines that play an important role in the physiology of pregnancy and the subsequent delivery (Casais *et al.* 2001). Finally, in the analysis of the neural and endocrine joint effect of NA and A_2 on the ganglion, a constant significant decrease in the release of ovarian progesterone was observed compared with A_2 alone. Although NA in the ganglion tends to diminish the luteotrophic effect of A_2, it is evident that the action of A_2 prevails. Future study of apoptosis at ovarian level should be developed to explain the molecular mechanism of ganglionic A_2 action.

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