RESEARCH

Sympathetic innervation regulates macrophage activity in rats with polycystic ovary

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Abstract

Polycystic ovarian syndrome (PCOS) is a low-grade inflammatory disease characterized by hyperandrogenism and ovarian hyperinnervation. The aim of this work is to investigate whether *in vivo* bilateral superior ovarian nerve (SON) section in adult rats with estradiol valerate-induced PCOS (PCO rats) affects macrophage spleen cells (MΦ) and modifies the steroidogenic ability of their secretions. Culture media of MΦ from PCO rats and PCO rats with SON section (PCO-SON rats) were used to stimulate *in vitro* intact ovaries. Compared with macrophages PCO, macrophages from PCO-SON rats released less tumor necrosis factor-α and nitric oxide, expressed lower *Bax* and *Nfkb* mRNA and showed reduced TUNEL staining. Also, in PCO rats, the SON section decreased kisspeptin and nerve growth factor mRNA expressions, without changes in *Trka* receptor mRNA levels. Macrophage secretions from PCO-SON rats decreased androstenedione and stimulated progesterone release in PCO ovaries, compared to macrophage secretions from PCO rats. No changes were observed in ovarian estradiol response. These findings emphasize the importance of the SON in spleen MΦ, since its manipulation leads to secondary modifications of immunological and neural mediators, which might influence ovarian steroidogenesis. In PCO ovaries, the reduction of androstenedione and the improvement of progesterone release induced by PCO-SON MΦ secretion, might be beneficial considering the hormonal anomalies characteristic of PCOS. We present functional evidence that modulation of the immune-endocrine function by peripheral sympathetic nervous system might have implications for understanding the pathophysiology of PCOS.

Key Words

- polycystic ovary
- macrophages
- kisspeptin
- superior ovarian nerve
- steroidogenesis

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Introduction

In mammalians, reproduction is a highly complex phenomenon regulated by cross-talk between the neuroendocrine and immune systems (ThyagaRajan & Priyank 2012, Procaccini *et al.* 2014). Few data are available, however, on the functional significance of the ovary peripheral innervation in the pathogenesis of reproductive endocrine disorders. In rats, most of the sympathetic fibers innervating the ovaries arise from the superior mesenteric/celiac plexus ganglia and reach the ovary through the ovarian plexus and the superior ovarian nerve (SON) (Burden 1985, Klein & Burden 1988). SON mainly contains noradrenergic fibers from the celiac ganglion, in addition to other neuropeptides, such as vasoactive intestinal peptide and neuropeptide Y (Dissen & Ojeda 1999). The SON fibers, travel along the suspensory ligament and are distributed in the perifollicular theca layer, in close relation with the theca internal cells, thus participating in the regulation of ovarian steroidogenesis and follicular

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development (Aguado 2002). The sympathetic nervous system enters the spleen by periarteriolar pathways and terminates in T-cell and macrophage areas (Straub 2004). In addition to the presence of adrenergic receptors in these immunocompetent cells, histological studies have demonstrated that sympathetic nerve endings contain norepinephrine (NE), among other neuropeptides (Mignini *et al.* 2003). Likewise, it has been reported that the β_2 adrenergic receptor located on macrophages exerts an anti-inflammatory effect by inhibiting nuclear factor κB (NFκB) activation and cytokine production induced by pro-inflammatory stimuli (Farmer & Pugin 2000), although other reports suggest that β_2 adrenergic receptor may promote a pro-inflammatory response by macrophages (Tan *et al.* 2007). NFκB has been recognized as a redox-sensitive transcription factor involved in the induction of pro-inflammatory response (Kabe *et al.* 2005), as well as a mediator of genes responsible for cellular proliferation and apoptosis (Puszynski *et al.* 2009).

In recent years, kisspeptin (kiss) has emerged as a key regulator of the mammalian reproductive axis. This peptide hormone, acts via the G-protein-coupled receptor (GPR54) and stimulates secretions of hypothalamic gonadotropin-releasing hormone neurons to control puberty onset and subsequent fertility (Clarke & Dhillo 2016, Wahab *et al.* 2016). The kiss/GPR54 system is expressed in the ovarian, endothelial and immune cells of rodents and humans and in rat celiac ganglion where they colocalize with tyrosine hydroxylase neurons (Gaytán *et al.* 2009, Ricu *et al.* 2012). Intraovarian kiss/GPR54 may be regulated by sympathetic nerve activity and, together with NE, participate in the regulation of follicular dynamics (Fernandois *et al.* 2017). Kiss is known to be sensitive to immune/inflammatory challenge conditions and transmits these signals into the central reproductive system. In fact, decreased expression of kiss mediates acute immune/inflammatory stress-induced suppression of gonadotropin secretion in female rats (Iwasa *et al.* 2008).

On the other hand, Oakley *et al.* (2011) suggests that the spleen may serve as an immune cell reservoir for the ovary and that splenic monocytes can be mobilized in a cyclic manner to the ovaries where they differentiate into macrophages. Macrophage-derived secretory products such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and nitric oxide (NO), among others, exert direct effects on endocrine ovarian cells (Wu *et al.* 2004). These signaling molecules modulate normal reproductive function but are also involved in the pathogenesis of reproductive chronic inflammatory disorders as polycystic ovarian syndrome (PCOS) (Xiong *et al.* 2011). PCOS is one of the most common gynecological endocrinopathies affecting women in reproductive age. This disorder is characterized by hyperandrogenism, anovulation and infertility and involves abnormalities of lipid and glucose metabolism (Lizneva *et al.* 2016). Although its etiology remains unknown, a potential contribution of the peripheral sympathetic system in the initiation and/or perpetuation of PCOS has been proposed (Stener-Victorin *et al.* 2005, Wojtkiewicz *et al.* 2014). In particular, rats injected with a single dose of estradiol valerate (EV) develop anovulation and acyclicity, form ovarian cysts, and an increased ovarian sympathetic outflow that is accompanied by elevated synthesis of nerve growth factor (NGF) (Lara *et al.* 2000). Besides its function as a trophic factor for peptidergic and sympathetic neurons, NGF can act as an immunomodulatory factor through its receptors p75NGFR and TrkA, which are expressed by lymphocytes and monocytes (Thorpe *et al.* 1987). In the EV PCOS model, the SON section has the potential to restore estrus cyclicity and the ovulatory capacity of the ovary, supporting the theory of sympathetic hyperactivity (Barria *et al.* 1993).

Our studies using an EV-PCOS rat model (PCO rats) and secretions of splenocytes in culture, which is a heterogeneous cell population that includes B and T lymphocytes, macrophages as well as other cells, have suggested a functional relationship at the peripheral level between the immune, neural and endocrine systems. The increase of splenocytes β adrenergic receptors 7 days after the SON section was shown to be related to changes in ovarian steroidogenesis when secretions of splenocytes were used to induce the release of progesterone and estradiol from the ovary as compared to control rats (Forneris *et al.* 2003, 2008). However, the participation of the sympathetic system in PCOS pathogenesis through the SON and its interaction with the immune system (splenic macrophages) are yet poorly understood. Thus, in this study, we investigated whether bilateral section of the SON in EV-induced PCO rats would: (1) modify the expressions of sympathetic activation (NGF and kiss) and pro-inflammatory (TNFα and NO) markers in macrophages and induce apoptosis in these immune cells and (2) affect the steroidogenic ability of PCO macrophages secretions and consequently modify the ovarian steroid response. Culture media of macrophages from PCO rats (PCO) and PCO rats with bilateral section of the SON (PCO-SON) were used to stimulate *in vitro* PCO ovaries.

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Materials and methods

Materials

Estradiol valerate (EV), RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Sigma. TRIzol reagent was obtained from Invitrogen/Life Technology. Estradiol and androstenedione were provided by New England Nuclear (Boston, MA, USA). Other reagents and chemicals were of analytical grade.

Animals and treatment

Virgin adult female Holtzman rats (180±20 g body weight) showing at least two regular 4-day cycles were used. They were kept under controlled temperature and lighting conditions (22–24°C, 12-h light:12-h darkness) with free access to tap water and food (Cargill, Buenos Aires, Argentina). 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 ed. (T Poole ed., 1999). All animal procedures were performed following protocols previously approved by the Animal Use and Care Committee of the National University of San Luis.

Sixty-three rats were used and distributed into three groups: PCO ($n=27$) and PCO-SON ($n=27$) for ovarian incubation, where nine rats of each group were also used to obtain macrophages from the spleen, and the Control ($n=9$) group, where the rats were only used to obtain spleen macrophages. To induce the PCO condition, 8-week-old rats were injected with a single i.m. dose of 2 mg of EV in 0.1 mL of corn oil as vehicle (PCO rats) (Brawer *et al.* 1986). The *in vivo* bilateral sectioning of the SON in PCO rats was performed 7 days before killing (PCO-SON), as described previously (Forneris *et al.* 1999). Briefly, rats were anesthetized with a mixture of 20 mg/kg ketamine and 100 mg/kg xylacine. The ovaries were exposed through bilateral dorsal incisions, the suspensory ligament enclosing the SON was lifted with fine forceps and the nerve was cut with small scissors. Immediately, the ovaries were returned to the abdominal cavity and the incisions were sutured. Control rats received an injection of the vehicle only. PCO, PCO-SON and Control rats were killed by decapitation 60 days after application of the EV (when cystic follicles appear) or the vehicle. Control rats were killed on estrus day. Trunk blood was collected and serum was stored at −20°C until androstenedione concentrations were measured.

Macrophage culture

The macrophage (MΦ) culture was performed with the spleens from PCO, PCO-SON and Control rats. The tissue was sectioned into small pieces and pressed through a sterile nylon screen (200-μm mesh) to obtain the total cell populations. Cells were collected by centrifugation (1000 rpm; 5 min) and resuspended in red blood cell lysis buffer (pH 7.2). Cell viability and counts were determined by the trypan blue exclusion method at 0.4%. Cell viability was about 90% in all trials. Subsequently, 3×10^6 viable cells/mL of medium were incubated in RPMI1640 medium supplemented with 10% (v/v) inactivated FBS, 1% (v/v) sodium pyruvate and antibiotics (50 μg/mL streptomycin and 50 IU/mL penicillin), defined as basal medium (BM). After incubation for 2 h at 37°C in 95% air–5% CO_2, non-adherent cells were removed. The adherent MΦ monolayer showed 90% of purity according to the morphologic analysis performed by Giemsa staining and nonspecific esterase staining (Figueroa *et al.* 2015). MΦ from PCO rats (PCO MΦ), from PCO rats with bilateral SON section (PCO-SON MΦ) and from Control rats (Control MΦ) were plated at a density of 1×10^6 cells/well, preincubated in 1 mL of BM for 24 h and subsequently cultured for 24 h. The respective culture media (also called MΦ secretions) were collected and used to stimulate ovaries from PCO (PCO ovaries) and PCO-SON rats (PCO-SON ovaries).

Ovary incubation

After sacrificing the rats, the PCO and PCO-SON ovaries were rapidly halved and preincubated in 1 ml of BM at 37°C in 95% air–5% CO_2. After 15 min, the incubation media were discarded and either 1 mL of BM or 1 mL of MΦ secretions (PCO MΦ or PCO-SON MΦ culture medium, respectively) was added. Incubation was continued for 3 h, the medium was removed and stored at −20°C until measuring hormone release contents.

Steroid assays

Androstenedione (A2) levels in serum, as estradiol (E2), A2 and progesterone (P4) released in the media from ovarian incubations, were determined by radioimmunoassay using specific antisera (Forneris & Aguado 2002). The assay sensitivity was less than 0.02 ng/mL for A2, 12 fmol/tube for E2 and 5 ng/mL for P4. In all cases, the intra- and inter-assay coefficient of variation was lower than 10%. The results were expressed as nanograms of P4

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and A2 per milligram of ovarian tissue (ng P4/mg tissue and ng A2/mg tissue, respectively), and as picograms of E2 per milligram of ovarian tissue (pg E2/mg tissue).

Nitrite assay

MΦ culture supernatants from PCO, PCO-SON and Control rats were analyzed for NO by assaying nitrite, using the Griess reagent (Bryan & Grisham 2007). The intra-assay coefficient of variation was lower than 10%. Also, nitrite was measured in the macrophage spleen cells (MΦ) supernatant treated with 1 mM L-N^G-Nitroarginine methyl ester, a water-soluble inhibitor of NO synthase, in the culture medium for 24 h. The results were expressed as millimoles of nitrite per milliliter (mmol/mL).

Determination of TNFα

MΦ cells from PCO, PCO-SON and Control rats were incubated for 24 h and TNFα was quantified on cell-free culture supernatants by an ELISA kit (Chemicon International USA) according to the manufacturer's instructions. Cytokine concentration was determined by extrapolation from the TNFα standard curve and expressed as pg/mL.

RNA extraction and semiquantitative RT-PCR

Total RNA was extracted from MΦ culture using TRIzol reagent. The semi-quantitative analysis of mRNA was performed using a one-step RT-PCR method (Access RT-PCR system, Promega). All components for RT and PCR were assembled in 50 µL reactions containing 5× reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 3 mM $MgCl_2$, 10 mM dNTP mixture, 1 µM of each gene-specific primers, 2 µg template RNA, 5 units of AMV reverse transcriptase and 5 units of Tfl DNA polymerase. The amplification of cDNA was performed under the following conditions: denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 1 min and extension at 72°C for 2 min. The reaction was completed with a final extension at 72°C for 7 min (thermal cycler). The primer sequences are presented in Table 1. The PCR products were resolved on 2% agarose gel electrophoresis containing 0.5 mg/mL GelRed. Band intensities of RT-PCR products were quantified using NIH Image software. The relative abundance of each band was normalized according to the housekeeping *Gapdh* gene. Thus, results were expressed as mRNA/*Gapdh* in arbitrary units.

Identification of apoptotic nuclei

The *in situ* localization of nuclei exhibiting apoptotic DNA fragmentation from PCO, PCO-SON and Control rats were kept in MΦ cultured for 24 h. The DeadEnd Colorimetric TUNEL System kit (Promega) was used according to the manufacturer's instructions. Briefly, at the end of the cell culture period, the overlying medium was removed and 1×10^6 macrophages were harvested after 0.2% trypsin (Sigma) treatment. The cells were washed with ice-cold PBS, centrifuged and mounted on silane-coated slides to develop the TUNEL technique. Under light microscopy, the number of TUNEL-positive cells per high-power field (×100) was counted. Cells showing dark brown staining from the colorimetric reaction were considered positive

Table 1 Primer sequences for RT-PCR.

Gene	Primer (5′–3′)	Product (bp)	Annealing temp (°C)
Tnfa	Forward: AAGTTCCCAAATGGCCTCCCTCTCATC Reverse: GAGGCTGACTTTCTCCTGGTATGAAA	485	60
Bax	Forward: ACTAAAGTGCCCGAGCTGAT Reverse: TTCTTCCAGATGGTGAGCGA	190	61
Bcl2	Forward: CACCCCTGGCATCTTCTCCTT Reverse: AGCGTCTTCAGAGACAGCCAG	519	61
Trka	Forward: TGCTGCTGCTGCTGATTCTAGG Reverse: AGGAATGAGGTTGTCGGTGGTG	716	61
Ngf	Forward: TGATCGGCGTACAGGCAGAAC Reverse: AAGGTGTGAGTCGTGGTGCAG	582	65
Nfkb	Forward: CATGAAGAGAAGACACTGACCATGGAAA Reverse: TGGATAGAGGCTAAGTGT AGACACG	329	59
Kiss	Forward: TGG CAC CTG TGG TGA ACC CTG AAC Reverse: GCC ACC TGC CTC CTG CCG TAG CGC	301	62
Gapdh	Forward: GGGCTGCCTTCTCTTGTGAC Reverse: CGCCAGTAGACTCCACGACA	325	60

bp, base pairs.

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for DNA fragmentation. A total of 50 fields were analyzed in all cases, and the result was expressed as a percentage of TUNEL-positive cells.

Statistical analysis

The results are expressed as the mean±standard error (S.E.M.). Statistical analysis was performed using GraphPad Prism version 5.0 for Windows. Student's *t*-test and ANOVA (parametric test) followed by the Tukey–Kramer test were used to compare the means between two groups for multiple comparisons. A value of $P<0.05$ was considered statistically significant.

Results

Effect of SON section on TNFα and nitrite release from PCO macrophages

The measurement of serum A2 levels showed a decrease in PCO-SON rats compare to PCO rats, reaching a similar value to control rats (PCO: 1.88±0.10; PCO-SON: 1.50±0.12; C: 1.45±0.07; ng/mL; PCO vs PCO-SON and C rats, $P<0.05$). This, along with our previous results showing that the androgen microenvironment induces the production of TNFα by PCO MΦ (Figueroa *et al.* 2015), led us to determine whether the SON section affects TNFα release and its mRNA expression in PCO MΦ. As shown in Fig. 1A and B, both the amount of TNFα released and its mRNA levels decreased in PCO-SON MΦ compared with PCO MΦ ($P<0.05$ and $P<0.01$, respectively), reaching a value near to control.

The results of nitrite released from MΦ are presented in Fig. 2. In a previous study, we observed that MΦ from PCO rats released more NO than Control MΦ (Figueroa *et al.* 2015). In this case, the SON section caused a decrease in the release of nitrites from PCO-SON MΦ in relation to PCO MΦ ($P<0.05$). When Control, PCO and PCO-SON MΦ were exposed to 1 mM L-NAME in the culture medium for 24 h, very low nitrite levels were observed ($P<0.001$) indicating that nitrite levels released from non-exposed MΦ can be associated to NO production.

Influence of PCO condition and SON section on the macrophage mRNA expression of *Bax*, *Bcl2* and *Nfkb*

Presented in Fig. 3A and B are the results of *Bax* (pro-apoptotic) and *Bcl2* (anti-apoptotic) mRNA levels

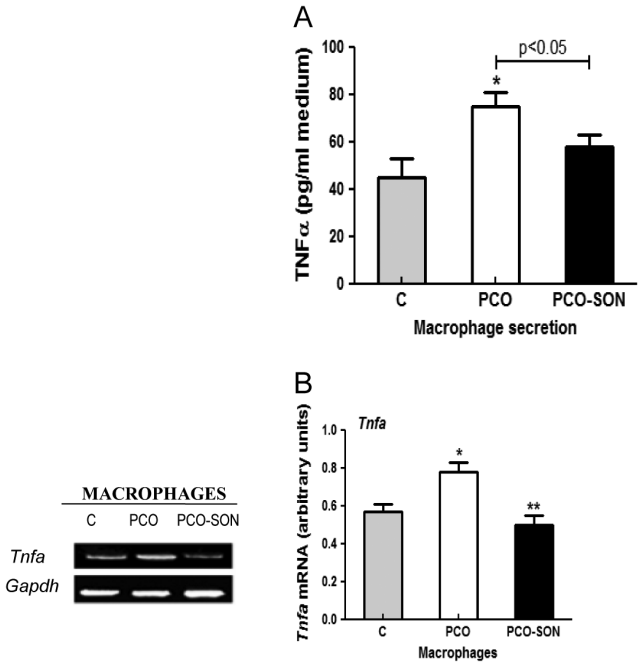

Figure 1
(A) Levels of tumor necrosis factor α (TNFα) released from spleen macrophages (MΦ) of Control rats (C), rats with polycystic ovary (PCO), and PCO rats with bilateral section of the superior ovarian nerve performed 7 days before killing (PCO-SON). MΦ were cultured with RPMI medium for 24 h. The graph represents the means±S.E.M. from three independent experiments with three rats per group. (B) *Tnfa* mRNA expression in MΦ from C, PCO and PCO-SON rats. Target mRNA (485 bp) was normalized by the level of *Gapdh* mRNA (325 bp). The bars represent the mean±S.E.M. of three independent experiments with three rats per group. *$P<0.05$ vs C MΦ and **$P<0.01$ vs PCO MΦ.

corrected for *Gapdh* expression in Control, PCO and PCO-SON MΦ. An increase of the *Bax* mRNA expression can be observed in PCO MΦ ($P<0.01$) compared with Control MΦ. PCO-SON MΦ expressed lower levels of *Bax* mRNA ($P<0.05$) in relation to PCO MΦ. As shown in Fig. 3B, no difference in the *Bcl2* mRNA expression was observed between PCO MΦ and Control MΦ; however, the levels of *Bcl2* mRNA in PCO-SON MΦ were higher than those in PCO MΦ ($P<0.05$). Based on these results, the *Bax*/*Bcl2* ratio decreased in PCO-SON MΦ compared to PCO MΦ ($P<0.01$). As shown in Fig. 3C, no significant change in *Nfkb* mRNA expression was observed between Control MΦ and PCO MΦ, while the SON section decreased *Nfkb* expression with respect to PCO MΦ ($P<0.01$).

Effect of SON section on the macrophages TUNEL staining in PCO condition

Figure 4 shows the apoptotic nuclei identified by the colorimetric TUNEL assay in control, PCO and PCO-SON

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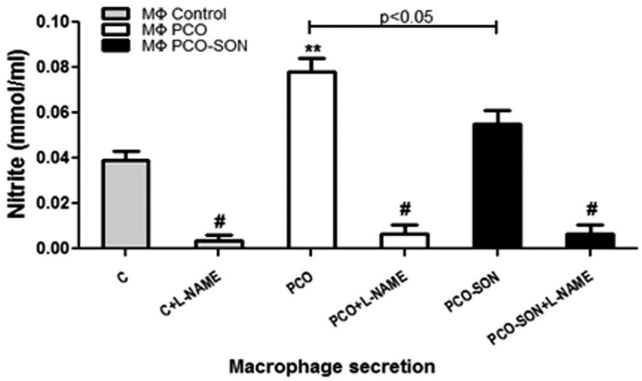

Figure 2
Levels of nitrites secreted from spleen macrophages (MΦ) of Control rats (C), rats with polycystic ovary (PCO), and PCO rats with bilateral section of the superior ovarian nerve performed 7 days before killing (PCO-SON). MΦ were cultured with RPMI medium, in presence or absence of nitric oxide synthase inhibitor (L-NAME), for 24 h. The graph represents the means ± S.E.M. from three experiments with three rats per group. #$P<0.001$ vs MΦ without L-NAME treatment and **$P<0.01$ vs C MΦ.

MΦ cultured for 24 h in RPMI medium. The number of apoptotic cells was higher in PCO MΦ compared to Control MΦ ($P<0.01$), and the SON section decreased TUNEL-positive cells in PCO MΦ ($P<0.05$).

Effect of SON section on *Ngf*, *Trka* and *Kiss* mRNA expressions in macrophages

It is known that NGF bound to its TrkA receptor stimulates *in vitro* the production of TNFα (Barouch *et al.* 2001). Figure 5A shows that PCO MΦ expressed higher levels of *Ngf* mRNA compared with Control MΦ ($P<0.01$), while PCO-SON MΦ showed lower neurotropin gene expression in relation to PCO and Control MΦ ($P<0.01$). As shown in Fig. 5B, no significant differences in *Trka* mRNA expression were observed between Control, PCO and PCO-SON MΦ. *Kiss* mRNA expression has been detected in peripheral blood leukocytes but no data are available of this peptide expression in rat spleen MΦ. As shown in Fig. 5C, *Kiss* mRNA expression was higher in PCO MΦ compared to Control MΦ ($P<0.05$), but it showed a significant decrease in PCO-SON MΦ as compared with PCO MΦ ($P<0.01$).

Effect of secretions of MΦ from PCO rats with and without SON section on the ovarian steroids release

In order to establish whether the SON section affects the steroidogenic ability of PCO and PCO-SON MΦ secretions, the ovaries from PCO and PCO-SON rats were incubated in the presence or absence of these secretions, followed by measurement of A2, E2 and P4 release to the incubation

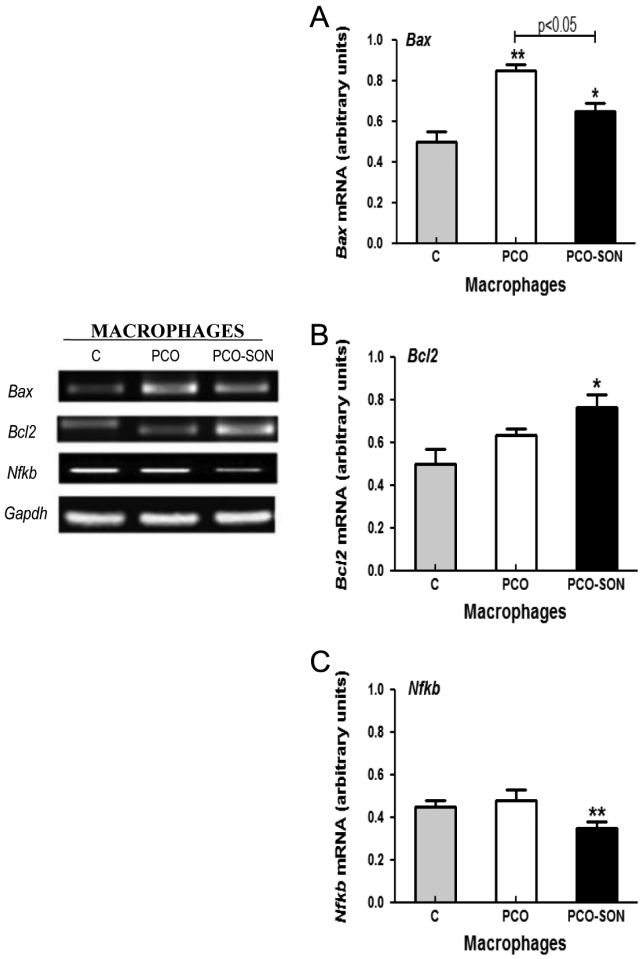

Figure 3
Expression of spleen macrophages (MΦ) genes from Control rats (C), rats with polycystic ovary (PCO) and PCO rats in which bilateral section of the superior ovarian nerve was performed 7 days before killing (PCO-SON). Representative RT-PCR analysis for (A) *Bax* (190 bp), (B) *Bcl2* (519 bp) and (C) *Nfkb* (329 bp). Macrophages were cultured with RPMI medium for 24 h. Target mRNA was normalized by the level of *Gapdh* mRNA (325 bp). Results are expressed as mean ± S.E.M. of three independent experiments, each experiment performed with three rats per group. *Bax*: *$P<0.05$ and **$P<0.01$ vs C MΦ; *Bcl2*: *$P<0.05$ vs PCO MΦ; *Nfkb*: **$P<0.01$ vs PCO MΦ.

medium. As shown in Fig. 6A, in basal conditions, A2 release from PCO-SON ovaries was not modified in relation to PCO ovaries. The secretions of PCO MΦ and PCO-SON MΦ had a stimulatory effect on A2 release from PCO and PCO-SON ovaries, compared to their respective basal values ($P<0.001$). In PCO ovaries, PCO-SON MΦ secretions induced a lower A2 release compared with PCO MΦ secretions ($P<0.01$), suggesting that the SON section modifies MΦ and, consequently, the steroidogenic ability of their secretion. The PCO-SON ovaries released less A2 with PCO-SON MΦ secretions compared to PCO ovaries ($P<0.05$). After stimulation of PCO-SON ovaries with the PCO MΦ secretions, the A2 release was decreased

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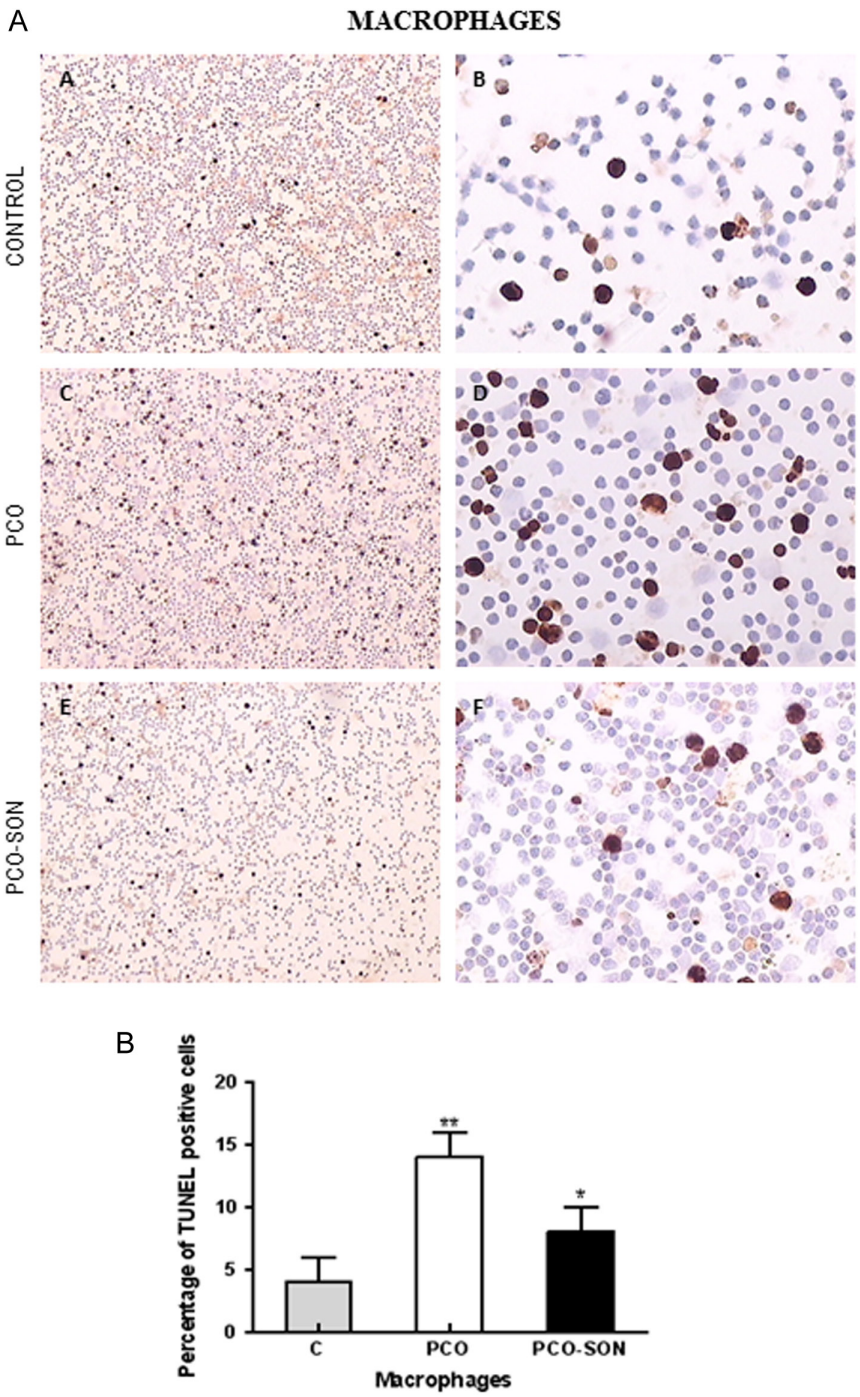

Figure 4
In situ detection of apoptosis by TUNEL in spleen macrophages (MΦ) of Control (C), PCO and PCO-SON rats, cultured for 24 h in RPMI medium. (A) Representative photomicrographs of the TUNEL reaction. Nuclear dark brown staining shows a positive reaction. Magnification A, C and E: 40×; B, D and F: 100×. Counter-staining: hematoxylin. (B) The bars indicate the number of apoptotic macrophage nuclei from three independent experiments with three rats per group; 50 fields per rat were analyzed. $^{**}P<0.01$ vs C MΦ; $^{*}P<0.05$ vs PCO MΦ. A full colour version of this figure is available at https://doi.org/10.1530/JOE-17-0736.

compared to PCO ovaries ($P<0.05$). However, the decrease of A2 release from PCO-SON ovaries with PCO-SON MΦ secretions was more pronounced with respect to PCO MΦ secretions ($P<0.01$).

Figure 6B shows that, in basal conditions, similar E2 levels were released by both PCO and PCO-SON ovaries. PCO MΦ and PCO-SON MΦ secretions induced higher E2 release from PCO and PCO-SON ovaries than their respective basal values ($P<0.001$ and $P<0.05$, respectively). In PCO ovaries, PCO-SON MΦ secretions did not significantly modify E2 release compared with PCO MΦ secretions. As it was observed with the release of A2, PCO-SON ovaries released lower E2 than PCO ovaries ($P<0.01$) in presence to PCO-SON MΦ secretions. After stimulation of PCO-SON ovaries with the PCO MΦ secretions, estradiol release was similar to that of PCO ovaries.

Figure 6C shows that, in basal conditions, P4 release from PCO-SON ovaries did not change in relation to PCO

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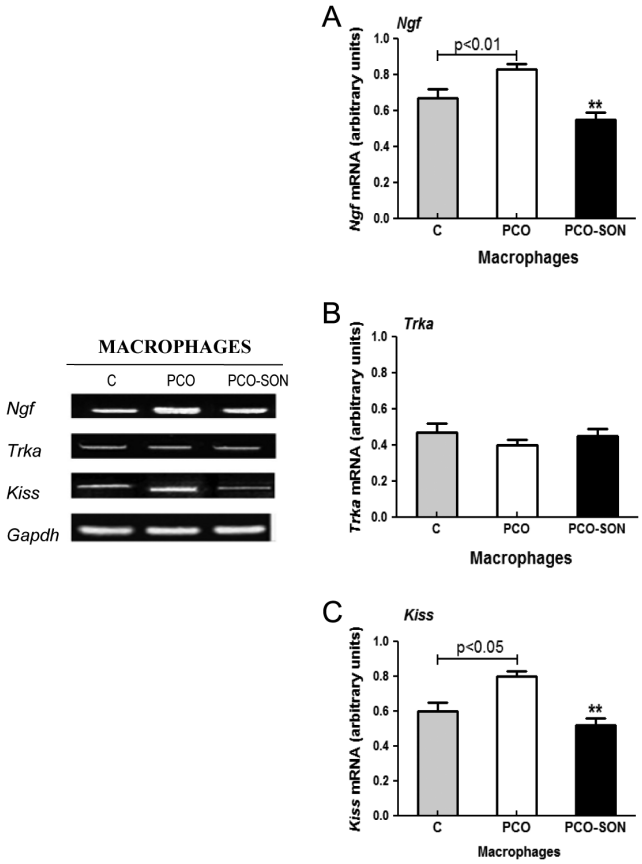

Figure 5
Spleen macrophages (MΦ) mRNA expression of (A) nerve growth factor (*Ngf*, 582 bp), (B) *Trka* receptor (716 bp) and (C) kisspeptin (*Kiss*, 301 bp). Macrophages from Control rats (C), rats with polycystic ovary (PCO) and PCO rats in which bilateral section of the superior ovarian nerve was performed 7 days before killing (PCO-SON). MΦ were cultured with RPMI medium for 24 h. Target mRNA was normalized by the level of *Gapdh* mRNA (325 bp). The bars represent the mean ± S.E.M. of three independent experiments with three rats per group. $^{**}P<0.01$ vs PCO MΦ.

ovaries. The secretions of both PCO MΦ and PCO-SON MΦ had a stimulatory effect on P4 release from PCO and PCO-SON ovaries, compared to their respective basal values ($P<0.001$). PCO ovaries released more P4 after stimulation with PCO-SON MΦ secretions in relation to PCO MΦ secretions ($P<0.01$). Besides, the P4 release from PCO-SON ovary incubated with culture medium from PCO-SON MΦ was lower compared to the corresponding PCO ovary ($P<0.05$). After stimulation of PCO-SON ovaries with the PCO MΦ secretions, the P4 release was decreased, in relation to PCO ovaries ($P<0.05$). The secretions of PCO-SON MΦ had a greater stimulatory effect on P4 release from PCO-SON ovaries than PCO MΦ secretions ($P<0.01$).

These data indicate that after SON section both macrophages and ovaries are compromised.

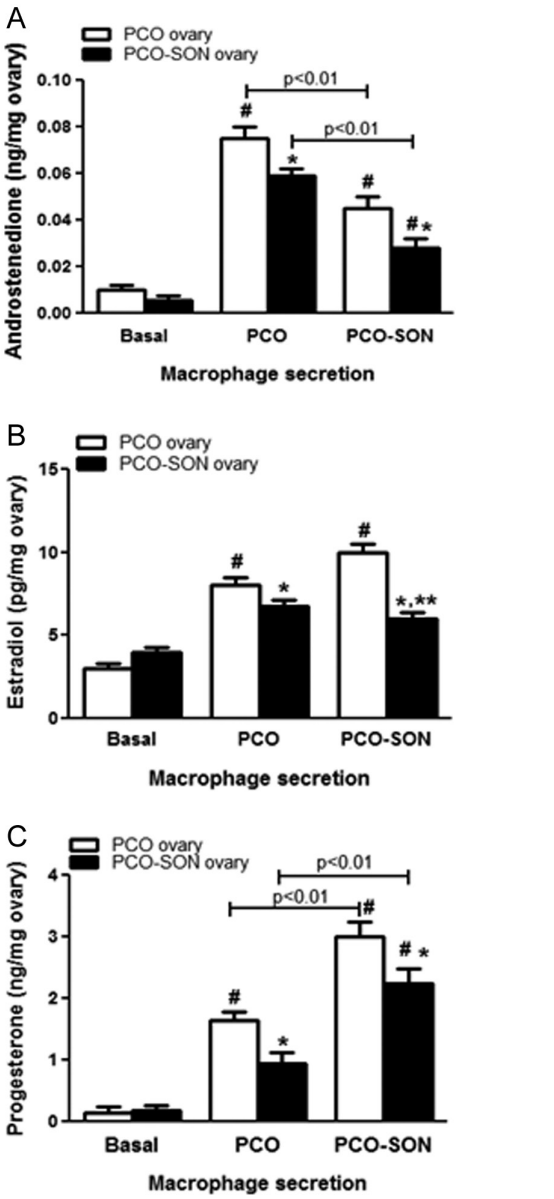

Figure 6
In vitro (A) androstenedione, (B) estradiol and (C) progesterone release from PCO and PCO-SON ovaries. Ovaries were stimulated with either basal medium (basal) or culture medium of PCO and PCO-SON macrophages (all being macrophage secretions) for 3 h. The graph represents the mean ± S.E.M. of three independent experiments, each experiment performed with three PCO and three PCO-SON rats. (A) $^{\#}P<0.001$ vs respective basal values, $^{*}P<0.05$ vs respective PCO ovaries; (B) $^{\#}P<0.001$ and $^{*}P<0.05$ vs respective basal value; $^{**}P<0.01$ vs respective PCO ovaries; (C) $^{\#}P<0.001$ vs respective basal values; $^{*}P<0.05$ vs respective PCO ovaries.

Discussion

There is growing interest in the complex relationship between the nervous, endocrine and immune systems in the regulation of reproduction and how its alteration

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can result in the development of illness. The PCOS is an endocrinopathy characterized by anovulation, infertility, hyperandrogenism and metabolic disorders in women of reproductive age. In addition to the compromise of the axis hypothalamus–hypophysis–ovary in PCOS, the contribution of the peripheral sympathetic system has been demonstrated by significant changes in the distribution pattern and density of catecholaminergic fibers in the ovary, in both human and animals (Stener-Victorin *et al.* 2005, Wojtkiewicz *et al.* 2014). The ovarian adrenergic fibers come from the SON, whose neuronal bodies are found in the celiac ganglion, where fibers that enter the spleen also originate (Bellinger *et al.* 1989). In the present study, we have shown in an EV-induced rat PCO model that the SON is involved in neuroimmunoendocrine regulation at peripheral level and that the bilateral section of the SON modifies the steroidogenic ability of splenic MΦ as well as expression of the neural and pro-inflammatory markers in these immune cells.

The nerve endings containing NE are distributed in specific compartments of the white pulp in the spleen and make direct contact with immunocompetent cells, which express adrenergic receptors (Mignini *et al.* 2003). Tan *et al.* (2007) have suggested that β_2 adrenergic receptor activation stimulates pro-inflammatory cytokine production in macrophages via PKA and NFκB independent mechanisms. Knowing that *in vivo* SON section modifies the number of splenocyte β adrenergic receptors (Forneris *et al.* 1999), it can be suggested that the decrease of mRNA expression of *Tnfa* as well as of TNFα release from PCO-SON MΦ may be related to lower NE levels reaching the spleen from the celiac ganglion. The possibility is not ruled out that other neurotransmitters or neuropeptides reaching the spleen may contribute to modulation of not only the TNFα release but also other cytokines release in MΦ. In this regard, Oliveros *et al.* (2001) have shown that the secretions of the cultured splenocytes from SON section rats produced a decrease of P4 and an increase of E2 release from intact ovaries. This steroidogenic response was reverted when ovaries were incubated with culture medium of SON sectioned rat splenocytes previously treated with vasoactive intestinal peptide or neuropeptide Y. This indicates that the spleen receives neuropeptides by neural route.

A potential role of the sympathetic nerve system in regulating inflammatory processes in PCOS has been proposed (Shorakae *et al.* 2015). Studies in cultured human *in vitro* fertilization-derived granulosa cells showed that NE and dopamine stimulate the generation of reactive oxygen species (Saller *et al.* 2014). In the present work, it was observed that the SON section decreases nitrite release and the mRNA expression of the inflammatory transcription factor *Nfkb* in PCO MΦ. Considering our previous reports (Forneris *et al.* 1999, Oliveros *et al.* 2001, Figueroa *et al.* 2012) and the data presented in this study, it can be suggested that the SON modulates *in vivo*, through the celiac ganglion in a retrograde way, the TNFα and NO release from PCO MΦ.

On the other hand, Liu *et al.* (2004) have shown that *in vitro* treatment of human MΦ with TNFα induces mitochondrial damage and promotes DNA fragmentation in association with inhibition of NFκB. We have shown here that the *Bax*/*Bcl2* ratio and the apoptotic nuclei number were increased in PCO MΦ compared with Control MΦ, indicating that PCO MΦs are more susceptible to apoptosis. This observation could be linked with the increase of TNFα and/or NO released by PCO MΦ. It has been reported that TNFα induces apoptosis in different cells types through NO production via upregulation of iNOS expression (Song *et al.* 2000, Sagoo *et al.* 2004).

Conversely, the SON section in EV-PCO rats reversed these apoptotic parameters in PCO MΦ and decreased the levels of TNFα released and its mRNA expression, as well as *Nfkb* mRNA levels. It is known that in some circumstances, activation of NFκB appears to sensitize cells to programmed cell death (Perkins 2007). Such reduction of apoptotic markers in PCO-SON MΦ could be related to a drop in NE release in the spleen after SON section. In fact, it has been reported that catecholamines exert a pro-apoptotic effect on lymphocytes *in vivo* (Stevenson *et al.* 2001) as well as on MΦ culture (Brown *et al.* 2003, Forneris *et al.* 2003).

It has been shown that neuropeptides such as NGF and kiss act as regulators of reproductive functions and can influence both the neuronal cell function (at the level of the central nervous system and peripheral system) and the inflammatory response (Sato *et al.* 2017, Minnone *et al.* 2017). Most inflammatory cells produce NGF and express TrkA. Thus, NGF induces the production of TNFα and IL-1β in macrophages under activation of TrkA (Minnone *et al.* 2017). Conversely, pro-inflammatory cytokines promote NGF synthesis in inflammatory tissues (Frossard *et al.* 2004). In this study, *Ngf* mRNA expression was higher in PCO MΦ in relation to Control MΦ, suggesting a role of the neurotropin in inflammatory processes. Studies have been reported on the pro-inflammatory properties of NGF/TrkA in murine MΦ as well as on the mechanism by which this complex stimulates *in vitro* TNFα and NO production, through activation of MAPK (Barouch *et al.* 2001). It is shown here that the SON section has

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an impact on PCO MΦ by decreasing the *Ngf* mRNA expression, which could contribute to reducing TNFα and NO synthesis in PCO MΦ.

On the other hand, Ricu *et al.* (2012) have suggested that the celiac ganglion is a site of synthesis of kiss, which is transported to the ovary through the SON, acting as a neurotransmitter in this organ. A similar pattern of mRNA expression of *Kiss* and *Ngf* was observed here. We have here found an increase of Kiss expression in PCO MΦ, which decreases after the SON section. These data may provide evidence that kiss in the spleen could affect macrophage activity. Although the immunomodulatory effects of kiss have not been fully explored yet, it has been reported that the levels of this peptide in plasma rise abruptly during normal pregnancy and have direct effects on the regulatory subpopulations of T lymphocytes (Gorbunova & Shirshev 2016). On the other hand, it has been demonstrated that *Kiss* mRNA expression is increased by TNFα, IL-6, MCP-1 and VCAM-1 in human endothelial cells, and thus, accelerates atherogenesis by enhancing the inflammatory responses (Sato *et al.* 2017). PCOS is associated with chronic low-grade inflammation and predisposition to hemostatic and atherosclerotic complications (Carvalho *et al.* 2017), accompanied by high plasma levels of kiss (Chen *et al.* 2010). In contrast, the decreased expression of kiss mediates acute immune/inflammatory stress-induced suppression of gonadotropin secretion in female rats (Iwasa *et al.* 2008).

It is known that PCOS is associated with hyperandrogenemia (Sung *et al.* 2014). In EV-treated animals, we observed that the serum A2 levels increased in PCO rats while the SON section decreased them. It is possible that the reduced number of TUNEL-positive nuclei in PCO-SON MΦ may be associated with decreased circulating A2 and hence with pro-apoptotic effect of androgens (Zhao *et al.* 2013).

Knowing that the SON is the main sympathetic pathway regulating steroidogenesis and follicular growth (Aguado 2002), we evaluated whether the SON section affects the steroidogenic capacity of macrophages in the ovary. The PCO-SON and PCO ovaries responded differently to stimulation with PCO-SON and PCO MΦ secretions. The PCO-SON ovaries stimulated with PCO-SON MΦ secretions released less A2 and P4 compared to PCO ovary. It must be highlighted that PCO-SON MΦ secretions elicited lower A2 release and higher P4 release from PCO ovaries in relation to PCO MΦ secretions, suggesting that the SON section modifies the steroidogenic ability of PCO MΦ secretions. Considering that TNFα production by PCO MΦ modifies ovarian response by increasing A2 levels (Figueroa *et al.* 2012), the steroidogenic effect of PCO-SON MΦ secretions could be associated to a lower release of TNFα by PCO-SON MΦ. In fact, it has been demonstrated that TNFα inhibits gonadotropin supported P4 production by murine, porcine and bovine ovarian cells (Korzekwa *et al.* 2008).

Women with PCOS have low levels of P4 related to oligo/anovulation-induced corpus luteum dysfunction as well as high spontaneous abortion rates. However, the mechanism underlying the reduced serum P4 in PCOS are not completely understood (Huang *et al.* 2016). The evidence obtained here shows that PCO-SON MΦ secretions improve the release of P4 from PCO ovaries, favoring in part the restitution of the luteal phase. Thus, we have shown that the SON section not only affects the MΦ activity but also modifies the ovarian response, observing a differential response to MΦ secretion of P4 release.

The results of our study provide further evidence of the importance of SON in the pathogenesis of the mechanisms underlying the PCOS. The SON bilateral section has the potential to improve the PCO condition in the studied

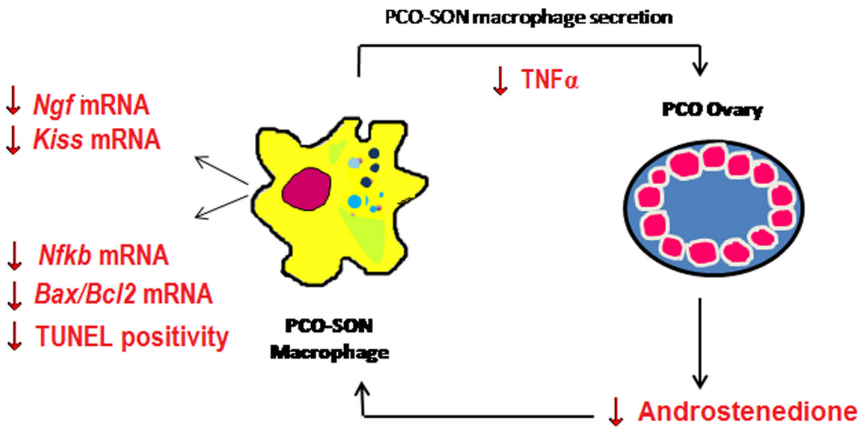

Figure 7
Schematic representation of the effects of superior ovarian nerve (SON) bilateral section on macrophages activity in rats with polycystic ovary (PCO). (↓: lower concentration than in the PCO group). A full colour version of this figure is available at https://doi.org/10.1530/JOE-17-0736.

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EV-treated animals, not only by the denervation effects directly on the ovary, as also observed by Morales-Ledesma *et al.* (2010), but also by the effects caused by denervation on the steroidogenic ability of spleen MΦ. The influence of the SON on MΦ activity is demonstrated here, since its manipulation led to modifications of immunological and neural mediators, which might affect ovarian steroidogenesis (Fig. 7). Additional investigations will be necessary to evaluate if the effects observed in this model are maintained in the long-term. Our results support the existence of a functional interaction between the immune, nervous and endocrine systems at peripheral level in PCOS.

Conclusions

The present study shows that ovarian denervation in PCO rats induced by SON section has an impact on PCO MΦ by decreasing the expressions of sympathetic activity markers as *Ngf* and *Kiss*, the release of pro-inflammatory molecules as TNFα and NO and apoptosis. In addition, the steroidogenic ability of MΦ secretions is demonstrated by the decrease of A2 release from PCO ovaries under stimulation with PCO-SON MΦ secretions. This suggests that the SON section, through its effect on MΦ, could improve the altered endocrine environment of PCO. In this work, we have demonstrated that the SON section could contribute to destabilizing the vicious cycle that maintains the hyperandrogenic state. It is possible to suggest that the SON regulates *in vivo* the macrophage activities of PCO rats. A better understanding of the neuroimmunoendocrine connection involving the SON, spleen MΦ and ovary in PCOS will permit to explore the controversial etiology of this disease and propose treatment options consistent with a biomedical approach.

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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