Immunohistochemical Study on Effectors of Ovary Medulla Blood Vessel in Pseudopregnant Rabbit

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ABSTRACT

Background: The ovarian blood flow provides oxygen-rich blood and nutrients which are necessary for the growth and secretory activities of follicles and corpora lutea (CL) in cycling and pregnant animals. The blood flow of the ovary is finely regulated by local and systemic mechanisms that integrate nervous, endocrine, and metabolic signals. In the rabbit ovary, administration of prostaglandin F2α (PGF2α) to pseudopregnant or pregnant animals affects specific vascular mechanisms regulating luteolytic process, in particular, the regulatory role of progesterone on luteal function through direct and uterine-mediated mechanisms. Thus, the main objective of the present work was to evaluate, at the level of ovarian blood vessels, the immunopresence of the receptors for progesterone (PR-R), PGF2α (FP) and GnRH (GnRH-R), and that of the enzymes endothelial nitric oxide synthase (eNOS), cyclooxygenase 1 (COX1), COX2, and prostaglandin E2-9-ketoreductase (PGE2-9-K).

Materials, Methods & Results: Sexually mature New Zealand White female rabbits were used for all experiments. The animals were housed individually, in an indoor facility under controlled conditions of light and temperature, and feed ad libitum. The rabbits were treated with equine chorionic gonadotropin followed 2 days later by an intramuscular injection of a GnRH analogue to induce pseudopregnancy. Five rabbits were sacrificed by cervical dislocation, at early, mid and late stages of pseudopregnancy, for the immunohistochemical detection of GnRH-R, COX1, COX2, PGE2-9-K, eNOS, FP, and PR-R. The ovaries were immersed for fixation in formaldehyde and subsequently processed for embedding in paraffin. Serial sections were cut and individually mounted on slides. Background labelling was prevented by incubating the sections with normal goat serum (for COX1, COX2, eNOS, GnRH-R, PR-R, and FP) and with normal rabbit serum (for PGE2-9-K). Subsequently, the sections were incubated overnight with the following primary antisera: mouse monoclonal anti-COX1, anti-COX2, anti-eNOS, anti-GnRH-R, and anti-PR-R, rabbit polyclonal anti-FP, and goat polyclonal anti-PGE2-9-K (CBR1). The next day, the slides were treated again with normal goat or rabbit serum and then incubated with biotin goat anti-mouse (for COX1, COX2, eNOS, PR-R, and GnRH-R), biotin goat anti-rabbit (for FP) or biotin rabbit anti-goat (for PGE2-9-K) secondary antibodies diluted 1:200 in TBS for 30 min at room temperature. The slides were exposed to the avidin-biotin complex and the peroxidase activity sites were visualized using the 3,3-diaminobenzidine tetrachloride kit as chromogen. At all stages of pseudopregnancy, the veins and arteries, within the ovary hilus and medulla, showed a variable immunoreactivity to anti-COX2, -PGE2-9-K, -eNOS and -FP antibodies, whereas anti-GnRH-R, -COX1 and -PR-R did not produce immunosignals. In particular, COX2 immunopositivity was moderately evidenced in the nuclei and cytoplasm of endothelial, smooth muscle and stromal cells of both veins and arteries. Strong positive immune reaction for FP was detected in the endothelial cells but not in the veins. PGE2-9-K immunosignals were moderately detected in the nuclei of endothelial, smooth muscle and stromal cells of both veins and arteries. The eNOS immunopositivity was moderately evidenced in the nuclei and cytoplasm of endothelial, smooth muscle and stromal cells of both veins and arteries. Strong positive immune reaction for FP was detected in the endothelial cells of ovarian arteries and veins; the arterial muscle cells were negative. Discussion: The present results suggest that the ovarian blood flow of rabbits is dynamically regulated as a consequence of exogenous treatments for the synchronization of estrous and the induction of ovulation. These haemodynamic changes are likely due to combined actions by several intraovarian factors, such as prostaglandins and correlated enzymes, steroids, and other autocrine/paracrine factors (GnRH, PGF2α, NO).

Keywords: rabbit, ovary, immunohistochemistry, COX, NOS, GnRH, prostaglandins, progesterone.
INTRODUCTION

The ovarian blood flow provides oxygen-rich blood and nutrients which are necessary for the growth and secretory activities of follicles and corpora lutea (CL) in cycling and pregnant animals [19]. The strict relationship between ovarian blood flow and development of ovarian structures is confirmed by the increase of vessel number and caliber [19]. In rabbits, the vascular network of the ovary has been studied by scanning electron microscopy of vascular corrosion casts throughout the different stages of estrus, pseudopregnancy, and pregnancy [7].

The ovarian blood flow is finely regulated by local and systemic mechanisms that integrate nervous, endocrine, and metabolic signals. Several studies have been carried out to examine whether specific vascular mechanisms in the rabbit ovary were implicated in the luteolytic process following administration of prostaglandin (PG) F2α (PGF2α) to pseudopregnant or pregnant animals [4,6,8-11,13-15,21,22,24]. Blood flow regulation within the rabbit CL and its relationship with steroidogenesis has also been investigated [16,20], suggesting that progesterone has a regulatory role on luteal function through direct and uterine-mediated mechanisms in pseudopregnant rabbit.

Thus, the main objective of the present work was to evaluate, at the level of ovarian blood vessels, the immunopresence of the receptors for progesterone (PR-R), PGF2α (FP) and GnRH (GnRH-R), and that of the enzymes endothelial nitric oxide synthase (eNOS), cyclooxygenase 1 (COX1), COX2, and prostaglandin E2-9-ketoreductase (PGE2-9-K)

MATERIALS AND METHODS

Animals, ovariopexy and hormonal regimen

Sexually mature New Zealand White female rabbits, weighing 3.5-4 kg, raised in premises owned by the University of Perugia, were used for all experiments. The protocol for the care and use of the animals for these experiments was approved by the Bioethics Committee of the University of Perugia. The rabbits were housed individually in an indoor facility under controlled conditions of light (14 h light/10 h darkness) and temperature (20-22°C). The animals were provided commercial rabbit chow and drinking tap water ad libitum.

The rabbits were treated with 20 IU of equine chorionic gonadotropin (eCG)1, followed 2 days later by an intramuscular injection of 0.8 μg of GnRH analogue2 to induce pseudopregnancy [18]. The day of GnRH injection was designated as day 0.

Immunohistochemistry of GnRH-R, COX1, COX2, PGE2-9-K, eNOS, PR-R and FP

All reagents were purchased from Sigma-Aldrich if not otherwise specified and locally. For the immunohistochemical detection of GnRH-R, COX1, COX2, PGE2-9-K, eNOS, FP, and PR-R five rabbits were sacrificed by cervical dislocation at day 4 (early), 9 (mid) and 13 (late) of pseudopregnancy. The ovaries, immediately excised, were immersed for fixation in formaldehyde (4%, w/v) in phosphate-buffered saline (PBS, pH 7.4) for 24 h at room temperature, and subsequently processed for embedding in paraffin following routine tissue preparation procedures [23].

Serial sections were cut at 4 μm and individually mounted on slides. After deparaffinization, the sections were submitted to antigen retrieval for all antisera with the exception for eNOS. This was carried out by microwaving in citrate buffer solution (10 mM, pH 6) for COX1, COX2, PGE2-9-K, PR-R and FP and in EDTA pH 8.0 for GnRH-R at 700 W for 10 min and then cooled to room temperature. After rinsing with TBS (tris buffered saline), they were dipped in 3% H2O2 in methanol for 1 h to quench the endogenous peroxidase activity and rinsed in TBS. Background labelling was prevented by incubating the sections with normal goat serum (for COX1, COX2, eNOS, GnRH-R, PR-R, and FP) and with normal rabbit serum (for PGE2-9-K) diluted 1:10, for 1 h at room temperature.

Subsequently, the sections were incubated overnight at 4°C in a moist chamber with the following primary antisera diluted in TBS containing 0.2% Triton X-100 and 0.1% bovine serum albumin (BSA): mouse monoclonal anti-COX1α (1:50); mouse monoclonal anti-COX2 and anti-eNOSβ (1:200 and 1:50, respectively); mouse monoclonal anti-GnRH-Rα (1:50); mouse monoclonal anti-PR-Rβ (1:500); rabbit polyclonal anti-FPα; goat polyclonal anti-PGE2-9-K (CBR1)γ (1:500) [12].

The next day, the slides were rinsed three times in TBS (5 min each), treated again with normal goat or rabbit serum and then incubated with biotin goat anti-mouse (for COX1, COX2, eNOS, PR-R, and GnRH-R), biotin goat anti-rabbit (for FP) or biotin rabbit anti-goat (for PGE2-
9-K) secondary antibodies\(^9\) diluted 1:200 in TBS for 30 min at room temperature. After TBS washes, the slides were exposed to the avidin-biotin complex (ABC)\(^9\) for 30 min and rinsed again with TBS. The peroxidase activity sites were visualized using the 3,3-diaminobenzidine tetrachloride kit (DAB)\(^9\), as chromogen; then the specimens were rinsed twice with distilled water for 5 min each, and, in some cases, were counterstained with Mayer’s haematoxylin. Finally, the sections were dehydrated passing through graded ethanol (70, 95 and 100%), cleared in xylene and mounted with medium for light microscopy. Sections in which the primary antibodies were omitted or substituted by corresponding pre-immune gamma globulin were used for the negative control of unspecific staining.

Figure 1. A-H Immunohistochemistry of the blood vessels present in the hilus and ovary medulla of the rabbit. (Photo A) anti-COX2: arterial (A) endothelial cells are strongly immunoreactive whereas the veins (V) are immunonegative; luteal cells (LC) are positive. (Photo B) anti-PGE2-9-K: the nuclei of endothelial, smooth muscle and stromal cells of veins (V) and arteries (A) and the luteal cells (LC) are moderately immunoreactive. (Photo C) anti-eNOS: immunosignals are localized in the nuclei and cytoplasm of endothelial, smooth muscle and stromal cells of both veins (V) and arteries (A) and in luteal cells (LC). (Photo D) anti-FP: positive FP-immune reaction is visible in endothelial cells of arteries (A) and veins (V). (Photo E) anti-GnRH-R, (Photo F) anti-COX1 and (Photo G) anti-PR-R: the wall of the veins (V) and arteries (A) are immunonegative whereas luteal cells (LC) display an immunoreaction; (Photo H) control section in which the primary antibody is omitted: all the histological structures are avoid of immunolabelling. [Scale bars = 20 μm].

RESULTS

At all stages of pseudopregnancy, the veins and arteries, within the ovary hilus and medulla, showed a variable immunoreactivity to anti-COX2, -PGE2-9-K, -eNOS and -FP antibodies (Figure 1. A-D) whereas anti-GnRH-R, -COX1 and -PR-R did not produce immunosignals (Figure 1. E-G).

In particular, COX2 immunopresence was strongly localized only in the arterial endothelial cells but not in the veins (Figure 1. A). PGE2-9-K immunosignals were moderately detected in the nuclei of endothelial, smooth muscle and stromal cells of both veins and arteries (Figure 1. B). The eNOS immunopositivity was moderately evidenced in the nuclei and cytoplasm of endothelial, smooth muscle and stromal cells of both veins and arteries (Figure 1. C). Strong positive immune reaction for FP was detected in the endothelial cells of ovarian arteries and veins; the arterial muscle cells were negative (Figure 1. D). The control staining procedure failed to disclose appreciable reactivity at any of the abovementioned sites for each antibody (Figure 1. H).

DISCUSSION

The localization of eNOS immunosignals in endothelial, smooth muscle, and stromal cells of both veins and arteries of the ovary, as here demonstrated, clearly suggests that the vasodilator NO may have a role in the decrease of vascular resistance [17].

The receptor for the GnRH has been recently identified in several ovarian structures of the rabbit [25], but not in the blood vessels of the ovary suggesting that a direct action of the GnRH itself on ovarian blood flow regulation is unlikely.

As regard as the enzymes involved in prostaglandin synthesis, our results revealed the immunopresence of COX2 in the endothelial cells of the ovary hilus and medulla, whereas PGE2-9-K immunosignals were detected more diffusely in smooth muscle, stromal, and endothelial cells. The widespread distribution of FP receptors in the rabbit CL and their localization in endothelial cells of ovarian vessels, as here demonstrated for the first time, suggest that the PGF2\(\alpha\) may act locally to cause an acute vasoconstriction that reduces blood flow. The acute increase in vascular resistance and decrease of the blood flow within the ovary may be also caused by release of luteal vasoactive peptides, such as endothelin-I and angiotensin-II
following PG2α administration [1-3]. Vasorelaxation and vasoconstriction are mediated by a number of endothelial systems including prostaglandins [5,16], in this context, our findings indicate that PGF2α may have a direct function in regulating blood flow with a paracrine (by endothelial and stromal cells) and autocrine (by smooth muscle cells) mechanisms. In particular, it could be hypothesized that endothelial cells, through COX2 activity, produce PGF2α and also PGE2. This latter prostaglandin is converted into PGF2α by PGE2-9-K in endothelial cells, but also in stromal and smooth muscle cells.

Finally, the lack of progesterone receptor and of COX1 in the blood vessels of the ovary hilus and medulla, indicates that this steroid and this enzyme are not directly involved in the blood flow regulation.

Taken together, these findings suggest that the ovarian blood flow of rabbits is dynamically regulated as a consequence of exogenous treatments for the synchronization of estrous and the induction of ovulation. These haemodynamic changes are likely due to combined actions by several intraovarian factors, such as prostaglandins and correlated enzymes, steroids, and other autocrine/paracrine factors (GnRH, PGF2α, NO).

**REFERENCES**


