Inhibition of nitric oxide synthesis potentiates apoptosis in the rabbit corpus luteum

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Abstract

To determine if nitric oxide (NO) plays a role in corpus luteum (CL) physiology by affecting progesterone secretion or luteal apoptosis, an in-vitro pseudopregnant rabbit ovarian perfusion system was used to measure the effects of an inhibitor of NO synthesis, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), on progesterone secretion and corpus luteal apoptosis as measured by internucleosomal DNA breakdown. Pseudopregnant rabbit ovaries perfused in vitro with L-NAME did not demonstrate any significant differences compared with control ovaries in progesterone secretion. However, apoptosis, as measured by internucleosomal breakdown, was significantly increased in L-NAME-perfused CL compared with controls. While NO does not appear to directly affect progesterone secretion, there does appear to be a role for NO in CL maintenance, or a role for inhibition of NO production in CL regression.

Keywords: apoptosis, corpus luteum, nitric oxide, progesterone, rabbit

Introduction

The corpus luteum (CL) is essential for the initiation and maintenance of pregnancy. If pregnancy does not occur, the CL regresses, allowing a new cycle to begin. Little is known about the biology of the involution of the CL. Regression is primarily characterized by a decrease in function, measured by a dramatic decline in progesterone secretion. However, the mechanisms that control progesterone secretion are still poorly understood. When progesterone secretion is maximal, blood flow per weight through the CL is also maximal, reaching concentrations as high as 3500 ml/min per 100 g tissue (Dharmarajan et al., 1994). Although blood flow decreases significantly during luteal regression, this cannot fully account for the decline in progesterone secretion (Abdul-Karim and Bruce, 1973; Bruce et al., 1984; Dharmarajan et al., 1994). Recent studies in the rat, cow, sheep, monkey, human and rabbit have shown that physiological cell death or apoptosis occurs during CL regression (Orlicky et al., 1992; Juengal et al., 1993; Dharmarajan et al., 1994; Fraser et al., 1995; Shikone et al., 1996; Roughton et al., 1999). However, it is unclear how structural luteolysis is related to the decline in progesterone synthesis that signals functional regression (Juengal et al., 1993; Dharmarajan et al., 1994). Many studies support a role for the immune system in luteal regression. During regression, ovarian prostaglandins (Schlegel et al., 1988) and free radical generation increase (Riley and Behrman, 1991; Hesla et al., 1992). White cells, particularly macrophages, infiltrate the CL (Bagavandoss et al., 1988, 1990; Wang et al., 1992; Brannstrom and Norman, 1993; Brannstrom et al., 1994). There is evidence to suggest that macrophages may play a role in luteal regression by secreting cytokines that could inhibit steroidogenesis (Benyo and Pate, 1992) or cause physiological cell death (Albina et al., 1993; Brannstrom and Norman, 1993).
The control of luteal regression is still poorly understood. In the rabbit, if pregnancy does not occur, progesterone secretion begins to decline after day 11 of pseudopregnancy, reaching baseline concentrations by day 18 (Hilliard et al., 1974; Dharmarajan et al., 1988). Oestradiol produced by ovarian follicles is the primary luteotropin in the rabbit CL (Bill and Keyes, 1983). However, luteolysis occurs despite the continued presence of oestradiol. Exogenous treatment with oestradiol does not prolong luteal lifespan (Miller and Keyes, 1976; Bill and Keyes, 1983). The prolongation of luteal function in pregnancy requires the presence of an as yet unidentified placentally derived factor (Khel and Carlson, 1981; Nowak and Bahr, 1983). The occurrence of apoptosis at the time of luteal regression (Dharmarajan et al., 1994) requires the activation of a specific endonuclease and implies a genetically regulated process (Schwartzman and Cidlowski, 1993). This contrasts with necrosis, which is a non-specific, non-genetically regulated process in response to various insults such as hypoxia or toxins (Schwartzman and Cidlowski, 1993). Although apoptosis occurs at the time of functional luteal regression, the proportion of luteal cells undergoing apoptosis cannot fully account for the dramatic decline in progesterone secretion (Dharmarajan et al., 1994).

Nitric oxide (NO) is a simple gas with free radical chemical properties. Although it has been implicated in a number of diverse physiological processes that are crucial to luteal regression, such as regulation of blood flow, inhibition of steroidogenic enzymes and mediation of immune system effects, its role in CL physiology has not yet been examined. NO, also known as endothelium-derived relaxing factor or EDRF (Ignarro et al., 1987; Palmer et al., 1987), is a potent vasodilator (Moncada et al., 1991). It is not known whether NO plays a role in the regulation of luteal blood flow. Nitric oxide inhibits steroidogenesis in the adrenal gland, the testis and the ovary, probably by inactivating steroidogenic enzymes (Adams et al., 1992; Olson et al., 1996; Johnson et al., 1999; Dixit and Parvizi, 2001). On the other hand, many studies have shown that NO has both cytotoxic and cytoprotective effects. Studies investigating the role of NO in ovarian physiology have thus far only examined its effects during the periovulatory period. In the rabbit and rat, in-vitro and in-vivo studies support a role for NO in human chorionic gonadotrophin (HCG)-induced ovulation (Shukovski and Tsafiriri, 1994; Hesla et al., 1997). Finally, NO is released by activated macrophages during inflammatory processes, where it acts as a cytotoxic agent (Moncada et al., 1991). NO, released by macrophages infiltrating the CL, may play a role in luteolysis by causing cell death. Since NO may regulate processes which are crucial to CL regression, this study was undertaken to determine the role of NO in these processes.

In order to determine if NO is important in luteolysis, NO synthesis was inhibited in vitro in the CL using an arginine analogue N\textsuperscript{G}-nitro-l-arginine methyl ester (l-NNAME). The effects of the inhibition of NO generation were determined using two parameters: (i) progesterone secretion, which reflects luteal function, and (ii) the presence of internucleosomal DNA breakdown, which reflects physiologic cell death, a measure of structural regression.

Materials and methods

Animals

Sexually mature New Zealand White rabbits weighing an average of 3.5 kg were housed individually under controlled temperature and light and given free access to food (Purina Rabbit Chow;Ralston Purina Co., St Louis, MO, USA) and water. Rabbits received 100 IU HCG (Organon, West Orange, NJ, USA) via the marginal ear vein to induce pseudopregnancy. The day of injection was defined as day 0 of pseudopregnancy. On the appropriate day of pseudopregnancy, rabbits were anaesthetized with intravenous sodium pentobarbital (32 mg/kg), anticoagulated with heparin sulphate (120 IU/kg) and then subjected to laparotomy. In the rabbit, pseudopregnancy lasts for 21 days with progesterone secretion increasing from days 1 to 11 and declining to baseline concentrations by day 18 (Hilliard et al., 1968; Browning and Wolfe, 1981; Dharmarajan et al., 1988). In this study, the experiments were performed on day 18 of pseudopregnancy because in-vivo administration of the NO inhibitor L-NNAME from day 11 to day 18 did not effect progesterone production over a period of time (data not shown). All protocols were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee.

Preparation of ovaries for perfusion

The cannulation procedure and perfusion apparatus have been described in detail previously (Lambertson et al., 1976; Dharmarajan et al., 1988). Briefly, on day 18 of pseudopregnancy, rabbits received one dose of either intramuscular saline or 40 mg/kg L-NNAME. They were then anaesthetized with 32 mg/kg intravenous sodium pentobarbital. Heparin sulphate, 120 IU/kg, was given intravenously for anticoagulation. All rabbits underwent laparotomy after induction of anaesthesia. The anastomotic connections to each ovary were ligated. The ovarian artery and vein were isolated and dissected from the surrounding tissues and the artery and vein were individually cannulated. Both ovaries from each animal, with the vascular pedicles, were removed and placed in the perfusion chamber. The perfusion system consisted of a chamber containing the ovary, an oxygenator, a reservoir and a pulsatile roller pump that maintained perfusate flow at 1.5 ml/min, the approximate rate of blood flow to the rabbit ovary (Motta et al., 2001). The oxygenator was gassed with 95% O\textsubscript{2}/5% CO\textsubscript{2}.

In-vitro ovarian perfusion

Ovaries were perfused at 37°C for 6 h 15 min with 150 ml of Medium 199 (M.A. Bioproducts, Walkersville, MD, USA), pH 7.4, supplemented with 3% bovine serum albumin (BSA; Intergen, Purchase, NY, USA), heparin sulphate (200 IU/l), insulin (20 IU/l), streptomyacin sulphate (50 mg/l) and penicillin G (75 mg/l). Experimental ovaries were perfused in the presence of 0.01 mmol/l L-NNAME (Sigma, St Louis, MO, USA), while contralateral control ovaries were perfused with medium alone. Samples (1 ml) were obtained from arterial and venous sites at 0, 0.5, 1, 2, 4 and 6 h after time 0 (time 0 started 15 min after starting perfusion). Samples were stored at −20°C for later progesterone assay. Perfusate volume was maintained at 150 ml during the experimental procedure by replacing the perfusate samples with fresh medium.
Determination of internucleosomal DNA breakdown

After in-vitro perfusion of day 18 pseudopregnant ovaries for 6 h 15 min with or without 0.01 mmol/l L-NAME, ovaries were removed from perfusion chambers and placed on ice. Corpora lutea were immediately and carefully dissected out using micro-forceps. Isolated CL were snap frozen and stored at –70°C until DNA analysis could be carried out. Cellular DNA was isolated from CL and purified by phenol:chloroform extraction and ethanol precipitation. The DNA samples were quantified by optical density at 260 nm, labelled at the 3’ ends with (α32P)-dATP and resolved by gel electrophoresis. Following autoradiographic analysis, high (>15 kb) and low (<15 kb) molecular weight DNA fragments from each sample were excised from the gel and counted in a β-counter to estimate the degree of apoptotic DNA fragmentation. This procedure has been described previously in detail (Tilly and Hsueh, 1993; Dharmarajan et al., 1994).

Localization of DNA fragmentation

After perfusion for 6 h with 0.01 mmol/l L-NAME or control medium alone, ovaries were removed from the perfusion chamber and placed in 4% paraformaldehyde for 24 h. DNA fragmentation was assessed using a non-isotopic, streptavidin–biotin-based in-situ terminal transferase reaction previously described in detail (Gavieli et al., 1992; Tilly, 1994). Briefly, tissues were embedded in paraffin. Sections (5 μm) were transferred to glass slides. Sections were deparaffinized and digested with proteinase K (Boehringer-Mannheim, Indianapolis, IN, USA). DNA was labelled with terminal transferase enzyme and labelled nucleotides. After blocking with 3% BSA, streptavidin conjugated to alkaline phosphatase (Sigma) was added. The colorimetric reaction was carried out with nitroblue tetrazolium salt (Gibco-BRL, Grand Island, NY, USA) and 5-bromo-4-chloro-3-indolylyphosphate-toluidinium salt (Gibco-BRL). This reaction was terminated with 1 mmol/l EDTA/10 mmol/l Tris-HCl.

Progesterone assay

Progesterone concentrations in arterial and venous perfusate samples were measured using a commercial RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA) in which the progesterone antibody is bound covalently to the inner surface of polypropylene assay tubes. All samples and progesterone standards (100 μl) were assayed in duplicate. Samples from each experiment were assayed simultaneously to avoid error due to inter-assay variation. The intra-assay variation was 6.6%.

Ovarian progesterone secretion was calculated by dividing the mean of the differences in concentration between perfusate venous and arterial samples by the perfusion time. Progesterone secretion rates were compared by pairing control and L-NAME-treated ovaries from the same rabbit.

Experimental design

On day 18 of pseudopregnancy, rabbits underwent laparotomy and ovaries were perfused for 6 h with 0.01 mmol/l L-NAME or medium alone. During in-vitro perfusion, 1 ml perfusates were collected from arterial and venous sites simultaneously at 0, 30 min, 1 h, 2 h, 4 h, 6 h after time 0 (time 0 started at 15 min after beginning perfusion). At the end of the perfusion period, the ovaries were removed and the CL were collected and immediately snap frozen in liquid nitrogen and stored at –70°C until analysed for occurrence of internucleosomal DNA breakdown or placed in 4% paraformaldehyde for in-situ localization of DNA fragmentation.

Statistics

Results were compared by using Student’s t-test. Paired values from a single rabbit were compared using a paired t-test. P-values <0.05 were considered significant.

Results

Effect of L-NAME on progesterone secretion

On day 18 of pseudopregnancy (at the time of luteal regression), isolated, intact ovaries were perfused with 0.01 mmol/l L-NAME while contralateral ovaries from the same rabbit were perfused with control medium alone. Mean progesterone secretion rate in control ovaries was 1.45 ± 0.08 μg/h per ovary (n = 10). This rate is consistent with progesterone secretion rates on day 18 of pseudopregnancy in previous studies of in-vitro perfused rabbit ovaries (Dharmarajan et al., 1988). Mean progesterone secretion rate in ovaries perfused with L-NAME was 1.38 ± 0.22 μg/h per ovary (n = 10). Using a paired t-test to compare progesterone secretion rates between control and treated ovaries in the same rabbit, there were no statistically significant differences between the two groups (P = 0.16, Figure 1).

Effect of L-NAME on luteal internucleosomal DNA breakdown

Ovaries were perfused for 6 h 15 min with control medium or 0.01 mmol/l L-NAME. At the end of the perfusion, ovaries were removed from the perfusion chamber and individual CL were dissected out and snap frozen. Later, DNA was extracted from the isolated CL, labelled at the 3’ ends and resolved on agarose gels.

Perfusion with L-NAME significantly increased luteal internucleosomal breakdown. The DNA from CL in both control and L-NAME-perfused ovaries showed evidence of apoptosis, with resolution of DNA into the characteristic ladder pattern. The ladder pattern is formed by DNA fragments in multiples of 185 base pairs. Necrosis, as evidenced by DNA smearing, was not seen (Figure 2A).

The low molecular weight bands (less than 15 kb) were cut from the gel and radioactivity from end-labelling was quantified using a β-scintillation counter. Results were expressed in counts per minute of radioactivity (cpm). End-labelling of low molecular
weight bands in the CL perfused with L-NAME was significantly increased over control ovaries perfused with medium alone. Mean cpm in L-NAME-perfused CL was 5829.33 ± 19.27, while mean cpm in control M199-perfused CL was 1369.33 ± 32.26 (P = 0.0001). Low molecular weight DNA in CL perfused with M199 alone (control, mean cpm = 1223.33 ± 54.87, P = 0.1004). Fold changes in cpm were calculated with cpm from non-perfused day 18 CL set at 1.0. Low molecular weight DNA labelling from L-NAME-perfused CL was increased 4.79-fold over day 18 non-perfused CL (time 0), while low molecular weight DNA labelling from M199-perfused ovaries was increased only 1.12-fold over non-perfused CL (Figure 2B).

Localization of DNA fragmentation

Ovaries perfused for 6 h 15 min with and without L-NAME were removed from the perfusion apparatus and fixed in 4% paraformaldehyde. In-situ localization of 3' ends of DNA fragments showed evidence of DNA fragmentation primarily in the CL. The stroma showed little evidence of apoptosis. Staining for DNA fragments was present in luteal cells, and was intense in endothelial cells associated with the CL (Figure 3).

Figure 1. Effect of L-NAME on progesterone secretion. Progesterone secretion in μg/h per ovary in ovaries perfused with medium alone (control) paired with contralateral ovaries from the same rabbit perfused with L-NAME (n = 10 rabbits). Mean secretion rate in control ovaries was 1.45 ± 0.08 μg/h per ovary. Mean secretion rate by L-NAME-treated ovaries was 1.38 ± 0.22 μg/h per ovary. L-NAME did not significantly affect progesterone secretion (P = 0.16).

Figure 2. Effect of L-NAME on luteal internucleosomal DNA breakdown. DNA was extracted from isolated corpora lutea (CL) on day 18 of pseudopregnancy before perfusion (time 0) and after perfusion for 6 h 15 min with M199 (control) or medium containing 0.01 mmol/l L-NAME (n = 3). (A) After labelling of 3' ends with 32P, DNA fragments in multiples of 185 base pairs was seen, indicating apoptotic cell death. A representative gel is shown. (B) Low molecular weight bands (less than 15 kb) were cut out from the gel and counted in a β-scintillation counter. Counts per minute from day 18 CL that were not perfused (time 0) were set at 1.0 and fold change from time 0 was calculated and graphed for DNA end-labelling in CL perfused with control medium (M199) or L-NAME. Apoptosis was significantly increased in L-NAME treated CL compared with CL perfused with M199 alone (P = 0.0001), while apoptosis in CL perfused with M199 was not significantly different from unperfused day 19 CL.
Discussion

This study set out to determine whether NO plays a role in luteal regression. There are several possible mechanisms by which NO could regulate luteolysis. Data in the rabbit and rat support a role for NO in ovulation (Bonello et al., 1996; Hesla et al., 1997), but whether or not NO affects post-ovulatory ovarian function has not fully been examined. Nitric oxide synthase (NOS) has been localized by immunohistochemistry to follicular granulosa cells in the rabbit (Hesla et al., 1997).

After ovulation, the granulosa cells and theca cells form the CL (Niswender and Nett, 1994). NO is a potent vasodilator mediating vascular tone in many systems throughout the body (Moncada et al., 1991). In order to investigate the effects of endogenous NO generation a specific inhibitor of NOS, L-NAME, was utilized in these experiments. It is possible to measure the activity of NOS using an indirect assay for radioactively labelled citrulline, which is produced in a 1:1 ratio with NO from labelled L-arginine. Although NOS has been localized to granulosa cells in the ovary of the rabbit (Hesla et al., 1997), it was not possible to measure NOS activity in the CL by the citrulline assay. However, the results clearly show a biological effect of treatment with L-NAME. The increase in apoptosis implies the activation of a specific endonuclease that cleaves DNA. It is unlikely that L-NAME caused a non-specific toxic effect, since DNA smearing or necrosis was not observed in L-NAME treated ovaries. When L-NAME was given intramuscularly to rabbits in doses sufficient to produce serum concentrations equivalent to doses utilized in vitro, L-NAME caused a significant decrease in mean arterial pressure and increase in heart rate (data not shown), indicating a physiologically effective dose. Future studies will attempt to directly measure ovarian blood flow changes in response to L-NAME infusion.

Previous in-vivo studies in the testis have shown that L-NAME increases steroidogenesis (Wink et al., 1993), providing evidence for negative regulation of steroidogenic enzymes by endogenous NO. The mechanisms underlying the decline in progesterone secretion seen in the regressing CL remain unclear. If NO also has a negative regulatory effect in the CL, it would be expected that inhibition of NO synthesis would prevent the decline in progesterone secretion which occurs with functional luteal regression. However, inhibition of NO synthesis with L-NAME did not significantly affect luteal steroidogenesis in vitro. Therefore, NO does not appear to play an important role in the control of progesterone secretion in the regressing CL. More recent studies indicate that NO can play a role not only as a modulator of steroidogenesis (Motta et al., 2001), but also as a survival factor in the ovary (Chun et al., 1995). The role of NO in the ovary has been controversial until now. Depending on the concentration, NO can either stimulate DNA fragmentation or inhibit DNA fragmentation in granulosa cells, and this has been shown in bovine follicles (Chun et al. 1995). These reports suggest that the concentration of intracellular NO could be a critical factor in cell survival and function. The dual effect of NO was absent in the rabbit because progesterone production did not change following in-vivo...
In conclusion, NO appears to act through multiple mechanisms of NO will result in loss of trophic support, leading to cell death. luteal NO regulates luteal vessel dilatation and that withdrawal occurring primarily in luteal endothelial and parenchymal cells. Therefore, it appears that NO is somehow important for the structural regression of the CL, it would be expected that inhibition of NO production should inhibit structural regression. However, the inhibition of NO synthesis by L-NAME caused an increase, rather than a decrease, in apoptotic cell death in the CL, with little effect on DNA breakdown in the remainder of the ovary. Inhibition of NO synthesis with L-NAME did not cause other types of cell death, such as necrosis, to occur as evidenced by the lack of DNA smearing on gel electrophoresis. Previous studies have shown that on day 18 of pseudopregnancy apoptosis is present, while it is not present in the growing CL (Dharmarajan et al., 1994). The inhibition of NO synthesis at the time of regression further increases apoptotic cell death. By in situ localization of DNA fragmentation, apoptotic cell death is occurring primarily in luteal endothelial and parenchymal cells. Therefore, it appears that NO is somehow important for endothelial and parenchymal cell support. It is possible that luteal NO regulates luteal vessel dilatation and that withdrawal of NO will result in loss of trophic support, leading to cell death.

In conclusion, NO appears to act through multiple mechanisms to exert its full effects on CL function in vivo. The failure of L-NAME, NOS inhibitor, to cause a significant increase in progesterone secretion after intraovarian perfusion in vitro clearly suggest that NO plays a survival factor role and NOS inhibitors, such as L-NAME are luteolytic.

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