Dual effects of nitric oxide in functional and regressing rat corpus luteum

A.B.Motta1, A.Estevez, T.Tognetti, M.A.F.Gimeno and A.M.Franchi

Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Serrano 669, 1414 Buenos Aires, Argentina

1To whom correspondence should be addressed. E-mail: amotta@sion.com

The present study investigated the effect of nitric oxide (NO) on the lifespan of the corpus luteum (CL). Using a competitive nitric oxide synthase (NOS) inhibitor, l-nitro arginine methyl ester (L-NAME, 600 µmol/l), and a long-life NO donor, diethyl-aminetriamine (DETA-NONOate, 10⁻⁸, 10⁻⁶ or 10⁻⁴ mol/l), we found that in ovaries from rats at the mid stage of CL development, endogenous NO increased both glutathione (GSH) and progesterone production. However, during prostaglandin F₂α (PGF₂α)-induced luteolysis NO acted as an intermediary molecule in the inhibitory effect of PGF₂α on GSH content. This was supported by the fact that in-vivo PGF₂α treatment enhanced nitric oxide synthase (NOS) activity. These results indicate that the NO could act with a dual action (protective or pro-oxidant) in CL development.

Key words: corpus luteum/glutathione/luteolysis/nitric oxide/prostaglandin

Introduction

Luteal regression or luteolysis is an example of a decrease in cell function, which is a normal and necessary event in the mammalian reproductive cycle. This mechanism is widely studied for its implication in early pregnancy dysfunction. Corpus luteum (CL) involution has been related to an increased generation of reactive oxygen species (ROS), e.g. O₂⁻ and H₂O₂ by the intact ovary (Behrman and Preston, 1989; Riley and Behrman, 1991; Sawada and Carlson, 1991). One consequence of the production of free radicals in ovarian tissue is lipid peroxidation (Kappus, 1985), which occurs within the plasma membrane of luteal cells and may be associated with the observed loss of gonadotrophin receptors, diminished cyclic AMP formation and, therefore, with a decrease in steroidogenic ability of the CL during involution (Auletta and Flint, 1988; Wang et al., 1991). Protection against ROS in cells is provided by enzymes (superoxide dismutase, catalase and glutation peroxidase), metabolites, e.g. glutathione (GSH) or antioxidant vitamins (Amin et al., 1992), and has been suggested to be endocrine-regulated (Laloraya et al., 1988; Amin et al., 1992; Sugino et al., 1993, 1998, 1999).

Nitric oxide (NO) has been shown to participate in numerous physiological processes which are essential for maintaining homeostasis (Ignarro, 1990; Moncada et al., 1991). Some evidence supports the involvement of NO in ovarian physiology; nitrates are secreted from mammalian ovarian dispersates (Ellman et al., 1993), NO synthase (NOS) has been located in granulosa cells by immunocytochemistry (Zackrison et al., 1996), NOS inhibitors block human chorionic gonadotrophin (HCG)-induced ovulation in the rat (Shukovski and Tsafiri, 1994), endogenously produced NO and NO-releasing agents have been shown to inhibit steroidogenesis in granulosa and luteal cells in both rat and human (Van Voorhis et al., 1994; Olson et al., 1996). Recently, the opposite action of the NOS substrate, l-arginine, and HCG on human CL viability (Vega et al., 2000) has been described. Some authors have pointed out that NO augments oxidative stress-mediated toxicity, yet in some cases, NO has been shown to protect cells from the cytotoxicity of oxidants (Hogg et al., 1995; Wink et al., 1995).

GSH is a tripeptide thiol present in animal and plant cells. It participates in many cellular functions, including DNA and protein synthesis, regulation of enzyme activity, both intracellular transport, and acts as a major antioxidant in cellular protection (Denke and Fanburg, 1989).

The relationship between GSH and toxicity mediated by NO has been investigated by other authors. NO has been shown to react with intracellular GSH to form S-nitrosoglutathione (Clancy et al., 1994); NO induces oxidative stress and glutathione metabolism in rodent and human cells (Luperchio et al., 1996), regulates GSH synthesis in bovine aortic endothelial cells (Moellerling et al., 1999) and stimulates cystein (limiting factor in GSH synthesis) uptake by bovine endothelial cells (Li et al., 1999).

In previous studies of the mechanism of CL regression, we reported that oxytocin increased ovarian PGF₂α production during the late phase of pseudopregnancy in rats (Motta et al.,...
1996). This action was mediated by enhancing ovarian NOS activity (Motta et al., 1997). We also found that during the luteolytic process, endogenous NO increased PGF_{2\alpha} synthesis (Motta and Gimeno, 1997). By means of intrabursa ovarian sac treatment with two competitive NOS inhibitors and a NO generator, we demonstrated that NO produced locally had an antisteroidogenic effect, diminishing serum progesterone concentrations and increasing ovarian PGF_{2\alpha} production (Motta et al., 1999). However, at the mid stage of CL development, ovarian NOS activity was unexpectedly found to be higher than during regression (Motta and Gimeno, 1997), concomitant with the highest ovarian GSH content at this stage (unpublished data).

Taken together, we studied the possible relationship between NO and ovarian GSH at the mid stage of CL development. We have proposed that, in ovarian tissue (with functional CLs), high concentrations of locally produced NO could be regulating GSH synthesis. In addition, we studied the role of NO during luteolysis induced by PGF_{2\alpha} and its effect on ovarian GSH production.

**Materials and methods**

**Animals**

The animal model used was the same as that described previously (Lahav et al., 1989). Briefly, immature (28–30 days) female rats of the Wistar strain were given 15 IU/rat of pregnant mare’s serum gonadotrophin (PMSG; Sigma Chemical Co, St Louis, MO, USA). We considered day 0 of pseudopregnancy to be 48 h post-injection. This procedure induced formation of CL that remained functional for 9 ± 1 days (as determined by progesterone radioimmunoassay). The rats were housed under controlled temperature (22°C) and illumination (14 h light:10 h dark, lights on at 05:00) and allowed free access to Purina rat chow and water ad libitum. Animals were anaesthetized with ether and killed by cervical dislocation. As in previous studies (Motta et al., 1996), ovarian tissues from rats at the mid stage of CL development (day 5 of pseudopregnancy, with highest serum progesterone concentrations) were used.

**Experimental protocol**

**PGF_{2\alpha}-induced luteolysis**

For PGF_{2\alpha}-induced luteolysis, the experimental protocol was the same (dose and time) as that described previously (Motta et al., 1999). Rats were injected i.p. with 3 mg/kg body weight of a synthetic PGF_{2\alpha} (ILIREN, Hoerscht Vet) in 0.5 ml of saline solution. After 2 h, animals were anaesthetized with ether and killed by cervical dislocation. Ovaries were rapidly removed for culture assays or held on ice and frozen until determination of NOS activity.

**Ovarian cultures**

Removal of ovarian tissue was performed as previously described (Dunnam et al., 1999). Ovarian tissues were removed and cut into quarters. They were then randomly and evenly distributed at four explants per well in a 24-well culture plate (Nunc multidish, USA) for 18 h in an incubator with a humidified chamber and 5% carbon dioxide at 37°C. Separate aliquots of culture media were collected for GSH and progesterone determination.

**GSH determination**

The GSH assay was carried out as described previously (Tietze, 1969). Briefly, ovarian tissues were incubated in Krebs Ringer phosphate buffer for 1 h in a Dubnoff metabolic shaker under an atmosphere of 5% CO_2 in 95% O_2 at 37°C. At the end of the incubation period, tissues were removed and media collected for GSH determination. In treated samples a competitive NOS inhibitor, L-nitro-arginine methyl ester (L-NAME, 600 \(\mu\)mol/l) was added to the incubation media. We have previously found that 600 \(\mu\)mol/l of L-NAME (EC_{50}) inhibits NOS activity in ovarian tissue (Motta and Gimeno, 1997). Samples were then incubated with NADPH, glutathione reductase (GR) and Ellman’s reagent (a sulphydryl reagent: 5,5’-dithiobis-2-nitrobenzoic acid) yielding a chromophoric product with a molar absorption at 412 nm. Results are expressed as pmol GSH/g ovarian tissue.

**Determination of progesterone**

Incubation medium for hormone analysis was collected and frozen at the end of the incubation period, tissues were removed and media collected for GSH determination. In treated samples a competitive NOS inhibitor, L-nitro-arginine methyl ester (L-NAME, 600 \(\mu\)mol/l) was added to the incubation media. We have previously found that 600 \(\mu\)mol/l of L-NAME (EC_{50}) inhibits NOS activity in ovarian tissue (Motta and Gimeno, 1997). Samples were then incubated with NADPH, glutathione reductase (GR) and Ellman’s reagent (a sulphydryl reagent: 5,5’-dithiobis-2-nitrobenzoic acid) yielding a chromophoric product with a molar absorption at 412 nm. Results are expressed as pmol GSH/g ovarian tissue.

**Statistical analysis**

Statistical analyses were carried out using the Instat program (GraphPAD software, San Diego, CA, USA). Analysis of variance and Student–Newman–Keuls tests were used for comparisons between value of groups. *P* < 0.05 was considered to be significant.

**Results**

**NO produced by ovarian tissue enhances ovarian glutathione concentrations**

We incubated ovaries from rats at the mid stage of pseudopregnancy (day 5) with the competitive NOS inhibitor, L-NAME. L-NAME significantly inhibited total GSH production (Figure 1a). In contrast, a long half-life NO donor (DETA-NONOate) significantly enhanced ovarian GSH concentrations only at 10^{-6} mol/l (Figure 1b). There was no effect at higher or lower doses.

**NO produced by ovarian tissue enhances ovarian progesterone concentrations**

The inhibition of endogenous NO production by 600 \(\mu\)mol/l of L-NAME significantly diminished progesterone production in ovarian tissue from rats at the mid stage of CL development (Figure 2a). The NO donor, DETA-NONOate at 10^{-4} and 10^{-6} mol/l, significantly enhanced ovarian progesterone production after 18 h of treatment. At the lowest dose of DETA-NONOate used (10^{-8} mol/l), no effect on ovarian progesterone production was observed.
Nitric oxide in the corpus luteum

Figure 1. Effect of L-nitro arginine methyl ester (L-NAME) and diethyl-aminetriamine (DETA-NONOate) on glutathione production by ovarian tissue. (a) Ovarian tissues from rats at the mid stage of pseudopregnancy were incubated in 1 ml of Krebs Ringer phosphate buffer for 1 h at 37°C with or without 600 µmol/l of L-NAME. (b) Ovarian tissues from rats at the mid stage of pseudopregnancy were removed and cut into quarters and randomly distributed at four explants per well in a 24-well culture plate. Ovarian explants were then incubated with or without DETA-NONOate (10^−8, 10^−6 and 10^−4 mol/l) in medium 199 for 18 h in an incubator with a humidified chamber and 5% carbon dioxide at 37°C. At the end of the incubation period, tissues were removed and media was collected for glutathione (GSH) determination. Each group represents the mean ± SEM of 10 different animals. Experiments were carried out in duplicate. *Significantly different from the other groups (P < 0.05).

PGF_2α diminishes glutathione produced by ovarian tissue: NO as a possible intermediary metabolite

As previously described (Motta et al., 1999), we induced luteolysis with a synthetic PGF_2α. After this procedure GSH production was diminished compared with that in controls (Figure 3). When L-NAME was added during the incubation period, PGF_2α-induced inhibition of ovarian GSH production was completely abolished (Figure 3).

PGF_2α induces luteolysis by increasing NOS activity

In order to know whether NO could be an intermediary in the action of PGF_2α on GSH depletion during induced luteolysis, we determined NOS activity in ovarian homogenates from rats injected with PGF_2α 2 h before the determination of NOS activity. As shown in Figure 4, NOS activity was significantly enhanced by the in-vivo PGF_2α treatment.

Discussion

When first discovered, NO was first considered to be a relaxation molecule (Ignarro et al., 1990; Moncada et al., 1991). Following this, many studies were conducted to investigate the role of NO in the oxidative stress, both in pathological and physiological events (Shukovski and Tsafiri, 1994; Van Voorhis et al., 1994; Motta et al., 1996, 1997). However, the formation of NO can lead to the production of reactive nitrogen oxide species (RNOS) (Wink et al., 1996). Like ROS, these NO-derived reactive intermediate species can result in damage to specific cellular targets, which leads to speculation that NO may have a significant role in various pathological conditions (Gross and Wolin, 1995; Wink et al., 1996). Recently, this radical gas has been shown to participate in numerous physiological processes which are essential for maintaining homeostasis (Ignarro, 1990; Moncada et al., 1991). Recent reports (Wink et al., 1995; Hogg et al., 1995) have pointed out that NO could protect cells from oxidants. Apparently, the toxic or protective effect of NO appears to be determined by intracellular redox status, as shown in rat oligodendrocytes (Rosenberg et al., 1999).

Taken together, and in view of the fact that we found...
Figure 3. Effect of L-nitro arginine methyl ester (L-NAME) and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on glutathione (GSH) production. Ovarian tissues from rats at the mid stage of pseudopregnancy were incubated with or without L-NAME (600 µmol/l) in 1 ml of Krebs Ringer Phosphate buffer for 1 h at 37°C. In another group, PGF$_{2\alpha}$ (3 mg/kg weight) was injected i.p. in rats at the mid stage of pseudopregnancy to induce luteolysis. After 2 h, ovarian tissue was removed and incubated with or without L-NAME, as described above. At the end of incubation period, tissues were removed and media collected for GSH determination. Each group represents the mean ± SEM of 10 different animals. Experiments were carried out in duplicate. *Significantly different from the other groups (P < 0.05).

Figure 4. Effect of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on the nitric oxide synthase activity. Rats at the mid stage of pseudopregnancy were i.p. injected with PGF$_{2\alpha}$ (3 mg/kg weight) for inducing luteolysis. After 2 h, the ovarian tissues were removed for determination of nitric oxide synthase (NOS) activity. Each group represents the mean ± SEM of 10 different animals. Experiments were carried out in duplicate. *Significantly different from the other groups (P < 0.05).

diminished NOS activity with the age of the CL (Motta et al., 1997), the present study was performed in order to investigate the possible protective function of endogenous ovarian NO in the early stage of CL development and to compare this role with its role during luteolysis.

In the present study, we found that NO produced by ovarian tissue at the mid stage of CL development increases GSH production, as demonstrated by the fact that the specific NOS inhibitor diminished, while a long half-life NO donor increased GSH concentrations. This latter effect was observed at $10^{-6}$ mol/l concentration of DETA-NONOate, while lower and higher doses had no effect. The failure of the higher dose ($10^{-4}$ mol/l) to stimulate GSH production could be the result of down-regulation on the NOS activity by a direct effect of NO (Rengasamy and Johns, 1993). We believe that, in the early stage of CL development, the chronic exposure of endogenous NO could be responsible for the increase in the ovarian GSH production. In the past, other authors have investigated the relationship between GSH and NO involved in a protective role, in different systems. Rosenberg et al. demonstrated that NO donors with longer half-lives were required for a protective effect in rat oligodendrocytes in culture (Rosenberg et al., 1999), while short-lived NO donors produced a cytotoxic effect depleting GSH content. These apparently controversial results could be explained by the fact that an acute exposure of cells to NO donors would lead to depletion of GSH, whereas chronic exposure would result in a progressive increase in cytosolic GSH (Li et al., 1999; Moelling et al., 1999; Rosenberg et al., 1999). In spite of diverse mechanisms being proposed (NO increasing cystine uptake, NO inducing γ-glutamylcysteine synthetase and γ-glutamyl transpeptidase), the activation of a crucial protective factor or inactivation of a crucial death factor in the setting of oxidative stress is still unknown.

Using L-NAME to inhibit endogenous NO and a long half-life NO donor (DETA-NONOate), we found that NO increased progesterone production. This result is in agreement with that of Dong et al. who demonstrated that NO reversed prostaglandin inhibition in ovarian progesterone secretion in rats (Dong et al., 1999). However, in contrast, a previous study using rat ovarian tissue during the late stage of CL development, demonstrated that in-vivo NO generator treatment (sodium nitroprusside) diminished serum concentrations of progesterone (Motta et al., 1999). Those results led us to conclude that NO can have opposing actions, depending on the stage of CL development. As in the GSH determination experiments, there appears to be a dose-dependent relationship between DETA NONOate and progesterone since $10^{-6}$ and $10^{-4}$ mol/l increased (while $10^{-8}$ mol/l had no effect) ovarian progesterone production.

As NO was able to increase progesterone production and ovarian GSH content (an antioxidant metabolite) we could assume a protective role for NO at the mid stage of CL development. Recently, a dual response to NO in the functional and regressing human CL has been proposed (Friden et al., 2000). These authors demonstrated that a NO donor (spermine NONOate) significantly decreased the production of progesterone in human CL cells of the late, but not the mid, luteal phase. In addition, these authors found that NO increased concentrations of both PGF$_{2\alpha}$ and PGE during the late luteal phase.

We also focused our study on the role of NO during luteolysis in order to compare the effect with the above-mentioned protective action. In previous reports, we had found that during CL demise, PGF$_{2\alpha}$ and NO were related by a positive feedback pathway leading to higher concentrations of lipid peroxidation (Motta et al., 1996) concomitantly with a diminution in the progesterone concentrations (Motta et al., 1999). In this study, we demonstrated that PGF$_{2\alpha}$ diminishes GSH content, and that this may be mediated by NO. The fact that PGF$_{2\alpha}$-treatment increased NOS activity also suggests that this action could be NO-dependent. In agreement with our previous report (Motta et al., 1999), this confirms that as in spontaneous luteolysis, the PGF$_{2\alpha}$ and NO/NOS system could be related during PGF$_{2\alpha}$-induced luteolysis.
In summary, we propose that during early stages of CL development, a chronic production of NO by ovarian tissue could lead to an increase in the GSH content in a protective role. On the contrary, during luteolysis, the highest and pulsatile ovarian PGF₂α secretion and acute NO production (Motta et al., 1996) would lead to an impairment in GSH production within the ovary. These results correlate well with previous findings that, during CL demise, PGF₂α (via NO) enhances lipid peroxidation (Motta et al., 2000). It is important to point out that the GSH metabolite is considered to be an important factor in the prevention of lipid peroxidation. To clarify the exact mechanism of NO regulation of ovarian GSH synthesis and its importance in oxidative stress is a motivation to further pursue the target(s) of NO activity to better understand the mechanism of CL regression.

Acknowledgements
These studies were supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 4076) and by Laboratorios Bagó, Argentina. The authors thank María Inés Casella and Ramona Morales for their technical support.

References

Received on July 18, 2000; accepted on November 1, 2000

**Nitric oxide in the corpus luteum**