Progesterone production in bovine luteal cells treated with drugs that modulate nitric oxide production

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The aim of this study was to investigate the influence of nitric oxide (NO) donors (*S*-nitroso-L-acetyl penicillamine, spermine–NO complex and sodium nitroprusside) and NO synthase inhibitors (*N*^ω-nitro-L-arginine methyl ester, *N*^ω-nitro-L-arginine, and (±)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine) on progesterone production by dispersed bovine luteal cells cultured for 24 h. All NO donors inhibited progesterone production and increased nitrite or nitrate concentration in the medium in a dose-dependent manner. Secretion of progesterone was reduced to 75% ($P < 0.01$), 56% ($P < 0.001$) and 37% ($P < 0.001$) by *S*-nitroso-L-acetyl penicillamine; to 65% ($P < 0.001$), 45% ($P < 0.001$) and 33% ($P < 0.001$) by spermine–NO complex and to 77% ($P < 0.05$), 74% ($P < 0.01$) and 54% ($P < 0.001$) by sodium nitroprusside treatments at concentrations of 10^{-5} , 10^{-4} and 10^{-3} mol l⁻¹, respectively, compared with the concentration of this hormone measured

in cells cultured in medium alone. NO synthase inhibitors decreased significantly ($P < 0.05$) nitrite or nitrate concentration and increased progesterone secretion with different potency at different doses. Significant increases in progesterone production were observed after *N*^ω-nitro-L-arginine methyl ester treatment at a concentration of 10^{-5} mol l⁻¹ and 10^{-4} mol l⁻¹, and after *N*^ω-nitro-L-arginine administration at a concentration of 10^{-6} mol l⁻¹ ($P < 0.01$) and 10^{-5} mol l⁻¹ ($P < 0.05$), compared with the concentration of this hormone measured in control cells. The results indicate that both NO donors and NO synthase inhibitors regulate steroidogenesis in cultured bovine luteal cells from days 10 to 14 of the oestrous cycle; however, the degree of progesterone inhibition by NO donors and stimulation by NO synthase inhibitors was dependent on the drug used.

Introduction

During the last decade many studies have shown that nitric oxide (NO), a highly reactive free radical, generated from L-arginine by nitric oxide synthase (NO synthase), acts as an important regulator of many physiological events (Moncada *et al.*, 1991). The NO synthase occurs in two functional classes that can be distinguished by their dependence on calcium for activity (Moncada *et al.*, 1991; Dong *et al.*, 1996). The constitutive isoforms NO synthase, NO synthase I (neuronal NO synthase) and NO synthase III (endothelial NO synthase) bind calmodulin in a reversible and Ca²⁺-dependent manner and release NO for short periods in response to receptor stimulation (Moncada *et al.*, 1991; Dong *et al.*, 1996). The cytokine-inducible isoform, NO synthase II (macrophage NO synthase) is calmodulin- and Ca²⁺-independent (Sessa, 1994; Dong *et al.*, 1996).

NO participates, with other substances, in the regulation of blood pressure (Ignarro, 1990), host defence (Bogdan, 2001; Eisenstein, 2001), neurotransmission (Kiss, 2000) and reproduction (Jaroszewski *et al.*, 2001). In the ovary, NO is involved in the process of follicular development and ovulation (Jablonka-Shariff and Olson, 1998; Drazen *et al.*, 1999), as well as corpus luteum function (Jaroszewski and Hansel, 2000; Skarzynski *et al.*, 2000). Many NO donors and NO synthase inhibitors have been used to determine the role of NO in progesterone production. It has been shown that the NO donors, *S*-nitroso-L-acetyl penicillamine and *S*-nitrosoglutathione, cause a dose-dependent decrease in progesterone secretion by human granulosa–luteal cells (Van Voorhis *et al.*, 1994). In contrast, NO synthase inhibitors, *N*^ω-nitro-L-arginine methyl ester and *N*^G-methyl-L-arginine, did not affect progesterone release (Van Voorhis *et al.*, 1994). Similarly, it has been shown that sodium nitroprusside, a NO donor, decreases progesterone secretion and aminoguanidine, but NO synthase inhibitor did not influence progesterone secretion in

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cultured cells obtained from luteinized rat ovaries (Olson *et al.*, 1996). In contrast, another NO donor (diethylenetriamine/NO) demonstrated a dose-dependent increase in progesterone production in ovaries collected from immature, equine chorionic gonadotrophin (eCG)-treated rats (Dong *et al.*, 1999). Recently, it has been shown that *S*-nitroso-L-acetyl penicillamine inhibited production of progesterone in dispersed bovine luteal cells (Skarzynski and Okuda, 2000). Perfusion of the bovine ovary with *N*^ω-nitro-L-arginine methyl ester increased progesterone secretion at either mid- or late-luteal phases (Jaroszewski and Hansel, 2000). However, addition of *N*^ω-nitro-L-arginine methyl ester and *N*^ω-nitro-L-arginine (another NO synthase inhibitor) increased progesterone production by dispersed bovine luteal cells from mid- but not from early cycle corpus luteum (Skarzynski and Okuda, 2000).

These conflicting data from differently designed experiments, performed under various conditions, led us to perform an experiment in which the influence of three nitric oxide donors (*S*-nitroso-L-acetyl penicillamine, spermine-NO complex and sodium nitroprusside) and three NO synthase inhibitors (*N*^ω-nitro-L-arginine methyl ester, *N*^ω-nitro-L-arginine and (±)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine) on progesterone production by bovine luteal cells, cultured in the same system and under the same conditions, could be compared.

Materials and Methods

Cell culture

Ovaries with corpora lutea were collected from cows at a local abattoir within 10–15 min after slaughter, placed in ice-cold saline and transported to the laboratory. The stage of the oestrous cycle was determined by macroscopic observation of the ovaries and the uterus (Miyamoto *et al.*, 2000) and corpora lutea were collected from ovaries and assigned to days 10–14 of the oestrous cycle. Enzymatic dissociation of the luteal tissue and the culture of luteal cells were performed as described by Skarzynski and Okuda (1999). Cell viability was greater than 85% as assessed by Trypan blue exclusion. The cell suspension contained about 20% large luteal cells, 70% small luteal cells and 10% endothelial cells or fibroblasts, but no erythrocytes.

The cells were adjusted to 1×10^5 viable cells ml⁻¹ of cultured medium: Dulbecco's modified Eagle's medium (DMEM) and F-12 Ham's medium (DMEM–Ham's F-12; 1:1 (v/v); Sigma, St Louis, MO) supplemented with 10% (v/v) calf serum (Sigma) and 20 µg gentamicin ml⁻¹ (Gibco Laboratories, Grand Island, NY). The cells were cultured in 48-well culture plates (Costar, Cambridge, MA) in a humidified incubator at 37.5°C in 5% CO₂ and 95% air atmosphere. After 24 h, the cells were washed twice with serum-free DMEM and then medium was replaced by fresh DMEM–Ham's F-12 supplemented

with 0.1% (w/v) BSA (Sigma), 0.5 mmol ascorbic acid l⁻¹ (Sigma), 5 ng sodium selenite ml⁻¹ (Sigma), 5 µg holo-transferrin ml⁻¹ (Sigma), and containing 20 µg gentamicin ml⁻¹. Simultaneously, the cells were treated for 24 h with various NO donors or inhibitors of NO production.

Experiment 1

The purpose of this experiment was to determine the effect of increasing doses of three NO donors on progesterone production and to evaluate the potency of each to cause NO liberation. In three separate replicates (two or three corpora lutea in each), the luteal cells were incubated with *S*-nitroso-L-acetyl penicillamine (10^{-5} to 10^{-3} mol l⁻¹; Sigma), spermine-NO complex (10^{-5} to 10^{-3} mol l⁻¹; Cayman Chemical Company, Ann Arbor, MI), sodium nitroprusside (10^{-5} to 10^{-3} mol l⁻¹; Sigma) or with bovine LH (USDA-bLH-B-6; 100 ng ml⁻¹) as a positive control.

Experiment 2

In this experiment, the effects of three NO synthase inhibitors (10^{-6} to 10^{-4} mol l⁻¹) on progesterone production and the potency of each to inhibition of NO releases were determined (three separate replicates, two or three corpora lutea each). The luteal cells were incubated with a non-selective NO synthase inhibitor (*N*^ω-nitro-L-arginine methyl ester; Sigma), an irreversible inhibitor of NO synthase I and III and reversible inhibitor of NO synthase II (*N*^ω-nitro-L-arginine; Sigma), a potent selective and reversible inhibitor of NO synthase II ((±)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; Sigma) or with LH (USDA-bLH-B-6; 100 ng ml⁻¹) as a positive control.

After 24 h of incubation, culture media were collected and stored at -20°C until assayed for progesterone and nitrite or nitrate.

Progesterone and nitrite or nitrate determinations

Progesterone in the culture media was assayed using a direct enzyme immunoassay as described by Okuda *et al.* (1997). The standard curve ranged from 0.39 to 100 ng ml⁻¹ and the effective dose for 50% inhibition (ID₅₀ value) of the assay was 4.5 ng ml⁻¹. The intra- and interassay coefficients of variation were 6.9% and 10.1%, respectively.

Nitrite or nitrate concentrations were determined using a colorimetric method (Griess Reagent; Green *et al.*, 1982). Briefly, nitrite standard (Sigma) was diluted in medium (DMEM–Ham's F-12) to a final concentration of 100 µmol l⁻¹ before determinations. Nitrate reductase (0.2 U (2 ml)⁻¹; Sigma) was dissolved in the dilution buffer (10 mmol glycine l⁻¹, 50 mmol Mops l⁻¹ at pH 7.2, 20% (v/v) glycerol and 0.1% (w/v) BSA; Sigma)

and NADPH (Sigma) was diluted in deionized water to a final concentration of 2 mmol l^{-1} . All solutions were protected from light. The assay was performed in a standard flat-bottomed 96-well polystyrene microtitre plate (Corning Inc., Corning, NY). Standards ($100 \mu\text{l}$ in serial dilution from 0 – $100 \mu\text{mol l}^{-1}$) or medium samples ($100 \mu\text{l}$) in duplicate, $10 \mu\text{l}$ of nitrate reductase solution (to convert nitrate to nitrite) and $10 \mu\text{l}$ of NADPH solution were added to each well, and the plate was swirled for 20 min at room temperature. Thereafter, $100 \mu\text{l}$ of Griess Reagent (Sigma; diluted in deionized water to the final concentration of 40 mg ml^{-1} directly before determination) was added to each well, mixed gently, protected from light and allowed to incubate for 15 min at room temperature. The absorbance was measured within 30 min using a 540 nm filter and plate reader (Bio-Rad, Hercules, CA). Concentrations of nitrite were determined using a standard curve constructed by plotting absorbance values versus nitrite standard concentrations. Sensitivity of the method was $0.085 \mu\text{g ml}^{-1}$ and the standard curve ranged from $0.05 \mu\text{g ml}^{-1}$ to $6.9 \mu\text{g ml}^{-1}$.

The intra- and interassay coefficients of variation were on average 8.2% and 14.9%, respectively.

Statistical analysis

Experimental data are presented as mean $\pm$ SEM of three separate experiments each performed in triplicate. The influence of NO donors on progesterone secretion and nitrite or nitrate concentration was described by a linear regression using a computer program GraphPad PRISM (GraphPad Software, San Diego, CA). The statistical significance of differences between controls and treated groups was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test (GraphPad PRISM). A value of $P < 0.05$ was considered to be significant.

Results

Nitrite or nitrate concentrations in the medium after luteal cell treatment with NO donors or NO synthase inhibitors

Increasing doses of all NO donors resulted in a dose-dependent increase of nitrite or nitrate concentrations, as shown by the linear regression ($r = 0.987$, $P < 0.001$; $r = 0.838$, $P < 0.001$ and $r = 0.779$, $P < 0.001$ for *S*-nitroso-L-acetyl penicillamine, spermine-NO complex and sodium nitroprusside, respectively). Significant increases in NO production were observed after treatment with *S*-nitroso-L-acetyl penicillamine at a concentration of $10^{-3} \text{ mol l}^{-1}$ ($P < 0.001$) and after administration of spermine-NO complex at $10^{-4} \text{ mol l}^{-1}$ and $10^{-3} \text{ mol l}^{-1}$ ($P < 0.001$) compared with controls

Fig. 1. Nitrite or nitrate concentrations in the medium after incubation of bovine luteal cells for 24 h in medium alone (control) or with LH and increasing concentrations of nitric oxide donors: *S*-nitroso-L-acetyl penicillamine (SNAP, ▨), spermine-nitric oxide complex (NONOate, ▩) and sodium nitroprusside (NP, ▧). Data are expressed as the means $\pm$ SEM of three separate experiments, each performed in triplicate. ^{a-c}Different subscripts indicate significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Con: control.

(Fig. 1). Moreover, the concentration of nitrite or nitrate in the medium after treatment of cells with spermine-NO complex at $10^{-4} \text{ mol l}^{-1}$ was significantly higher ($P < 0.001$) than after *S*-nitroso-L-acetyl penicillamine and sodium nitroprusside administration at the same concentrations. At the highest dose, spermine-NO complex released significantly more NO than did *S*-nitroso-L-acetyl penicillamine and sodium nitroprusside ($P < 0.001$) and *S*-nitroso-L-acetyl penicillamine released significantly more NO than did sodium nitroprusside ($P < 0.001$; Fig. 1). LH did not affect nitrite or nitrate concentration compared with controls (Fig. 1).

The changes in nitrite or nitrate concentrations in the medium collected after treatment of luteal cells with NO synthase inhibitors were less marked than after treatment with NO donors (Fig. 2).

Treatment with *N*^ω-nitro-L-arginine methyl ester decreased nitrite or nitrate concentration to 62% ($P < 0.001$), 54% ($P < 0.001$) and 64% ($P < 0.001$) of the control concentration at $10^{-6} \text{ mol l}^{-1}$, $10^{-5} \text{ mol l}^{-1}$ and $10^{-4} \text{ mol l}^{-1}$, respectively (Fig. 2). Increasing doses of *N*^ω-nitro-L-arginine decreased nitrite or nitrate concentrations to 73% ($P < 0.05$), 65% ($P < 0.001$) and 69% ($P < 0.01$), and ($\pm$)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine reduced nitrite or nitrate concentrations to 79% ($P > 0.05$), 62% ($P < 0.001$) and 69% ($P < 0.01$) compared with the concentration measured in cells cultured in medium alone (Fig. 2).

Fig. 2. Nitrite or nitrate concentrations in the medium after incubation of bovine luteal cells for 24 h in medium alone (control) or with LH and increasing concentrations of nitric oxide synthase inhibitors: *N*^ω-nitro-L-arginine methyl ester (L-NAME, ▨), *N*^ω-nitro-L-arginine (L-NOARG, ▩) and (±)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT, ▤). Data are expressed as the means ± SEM of three separate experiments, each performed in triplicate. ^{a,b}Different subscripts indicate significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Con: control.

Fig. 3. Secretion of progesterone by cultured bovine mid-luteal cells after incubation for 24 h in medium alone (control) or with LH and increasing concentrations of nitric oxide donors: *S*-nitroso-L-acetyl penicillamine (SNAP, ▨), spermine-nitric oxide complex (NONOate, ▩) and sodium nitroprusside (NP, ▤). Data are expressed as the means ± SEM of three separate experiments, each performed in triplicate. ^{a-f}Different subscripts indicate significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Con: control.

Secretion of progesterone by luteal cells incubated with NO donors

LH significantly increased ($P < 0.001$) progesterone secretion from cultured luteal cells after 24 h of incubation, indicating that cultured cells were react-

Fig. 4. Progesterone secretion from bovine mid-luteal cells cultured for 24 h in medium alone (control) or with LH and increasing concentrations of nitric oxide synthase inhibitors: *N*^ω-nitro-L-arginine methyl ester (L-NAME, ▨), *N*^ω-nitro-L-arginine (L-NOARG, ▩) and (±)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT, ▤). Data are expressed as the means ± SEM of three separate experiments, each performed in triplicate. ^{a-e}Different subscripts indicate significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Bonferroni's multiple comparison test. Con: control.

ive during the entire experimental period (Fig. 3). All nitric oxide donors inhibited progesterone production in a dose-dependent manner, as shown by the linear regression ($r = 0.723$, $P < 0.001$; $r = 0.599$, $P < 0.001$; $r = 0.624$, $P < 0.001$ for *S*-nitroso-L-acetyl penicillamine, spermine-NO complex and sodium nitroprusside, respectively). *S*-Nitroso-L-acetyl penicillamine inhibited progesterone production to 75% ($P < 0.01$), 56% ($P < 0.001$) and 37% ($P < 0.001$) of the control concentration after treatment with 10^{-5} mol l⁻¹, 10^{-4} mol l⁻¹ and 10^{-3} mol l⁻¹, respectively (Fig. 3). Increasing doses of spermine-NO complex caused a decrease in progesterone concentrations to 65% ($P < 0.001$), 45% ($P < 0.001$) and 33% ($P < 0.001$) of the concentration measured in cells cultured in medium alone (Fig. 3). The smallest reduction of progesterone production was observed after sodium nitroprusside treatment by 77% (10^{-5} mol l⁻¹, $P < 0.05$), 74% (10^{-4} mol l⁻¹, $P < 0.01$) and 54% (10^{-3} mol l⁻¹, $P < 0.001$) of the control concentration, respectively (Fig. 3). Spermine-NO complex at 10^{-4} mol l⁻¹ and 10^{-3} mol l⁻¹ reduced significantly more progesterone production compared with the same concentrations of sodium nitroprusside ($P < 0.05$; Fig. 3).

Secretion of progesterone by luteal cells incubated with NO synthase inhibitors

NO synthase inhibitors increased progesterone secretion, with different potencies at different doses (Fig. 4). *N*^ω-Nitro-L-arginine methyl ester increased progesterone secretion to 121% ($P > 0.05$), 190% ($P < 0.001$) and

167% ($P < 0.001$) at 10^{-6} mol l $^{-1}$, 10^{-5} mol l $^{-1}$ and 10^{-4} mol l $^{-1}$, respectively, compared with the control. Increasing doses of N^{ω} -nitro-L-arginine increased progesterone secretion to 146% ($P < 0.01$), 141% ($P < 0.05$) and 126% ($P > 0.05$) and ($\pm$)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine to 119% ($P > 0.05$), 138% ($P = 0.053$) and 123% ($P > 0.05$) compared with the control group. A higher stimulation of progesterone production was observed after N^{ω} -nitro-L-arginine methyl ester administration at 10^{-5} mol l $^{-1}$ ($P < 0.001$) and 10^{-4} mol l $^{-1}$ ($P < 0.01$) compared with the dose of 10^{-6} mol l $^{-1}$. Moreover, N^{ω} -nitro-L-arginine methyl ester at 10^{-5} mol l $^{-1}$ stimulated secretion of this hormone with a higher potency than the same dose of N^{ω} -nitro-L-arginine ($P < 0.01$) and ($\pm$)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine ($P < 0.001$). Similarly, N^{ω} -nitro-L-arginine methyl ester at 10^{-4} mol l $^{-1}$ caused a significantly higher increase in progesterone secretion than the same doses of N^{ω} -nitro-L-arginine and ($\pm$)-2-amino-5, 6-dihydro-6-methyl-4H-1,3-thiazine ($P < 0.05$; Fig. 4).

Discussion

The results of the present study demonstrate that all three NO donors inhibited progesterone secretion in a dose-dependent manner. Differences in the degree of inhibition of progesterone production by the NO donors were probably due to differences in the release of NO during cell culture, as reflected by nitrite or nitrate concentrations in the medium. Sodium nitroprusside, which releases NO at a lower concentration than S-nitroso-L-acetyl penicillamine and spermine-NO complex, was the least potent in inhibiting progesterone secretion. However, spermine-NO complex and S-nitroso-L-acetyl penicillamine were equally efficient in inhibiting progesterone secretion, even though spermine-NO complex was much more effective in releasing NO.

The inhibition of progesterone secretion by S-nitroso-L-acetyl penicillamine observed in the present study is in agreement with previous data of Skarzynski and Okuda (2000), which indicated a dose-dependent decrease of progesterone production in cultured bovine luteal cells. S-Nitroso-L-acetyl penicillamine also inhibits progesterone production in human granulosa-luteal cells (Van Voorhis *et al.*, 1994) and decreases gonadotrophin-stimulated progesterone production in ovaries from NO-deficient rats (Dunnam *et al.*, 1999). A similar inhibitory influence on progesterone secretion was observed after sodium nitroprusside addition to cells obtained from luteinized rat ovaries, incubated for 72 h (Olson *et al.*, 1996) and to rabbit corpora lutea collected at 4 and 9 days of pseudopregnancy (Gobbetti *et al.*, 1999). However, the inhibitory effect was greater in corpora lutea from day 9 than in those from day 4 of pseudopregnancy. The greatest decrease in progesterone production in our study was observed after treatment with spermine-NO

complex. Friden *et al.* (2000) showed that spermine-NO complex decreased progesterone secretion in human hCG-stimulated dispersed luteal cells; however, this effect was observed in cells from the late-, but not the mid-luteal phase.

As expected, the NO synthase inhibitors (N^{ω} -nitro-L-arginine methyl ester and N^{ω} -nitro-L-arginine) stimulated progesterone production by cultured bovine luteal cells. However, the increase produced by ($\pm$)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine was not statistically significant. Skarzynski and Okuda (2000) observed a similar stimulatory influence of N^{ω} -nitro-L-arginine methyl ester and N^{ω} -nitro-L-arginine on progesterone secretion. The strongest immunostaining for NO synthase III was observed in mid- and the late-luteal phase, and the highest immunostaining of NO synthase II was seen just before luteolysis (Skarzynski *et al.*, 2001). Because ($\pm$)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine is a potent selective NO synthase II inhibitor and the studies were conducted on corpora lutea from day 10 to day 14 of the ovarian cycle during which NO synthase III is localized in high quantity, lower stimulation of progesterone production after ($\pm$)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine stimulation compared with non-selective N^{ω} -nitro-L-arginine methyl ester may be a consequence of lower activity of NO synthase II in the mid-luteal cells compared with the late-luteal cells. The phase dependence of NO influence on progesterone production has been described in cattle (Skarzynski and Okuda, 2000), humans (Friden *et al.*, 2000), rats (Motta *et al.*, 2001) and rabbits (Gobbetti *et al.*, 1999). Moreover, results of many studies indicate that the mechanism controlling luteal regression in cattle is an immune cell- and immune response-dependent process (Pate and Landis Keyes, 2001; Petroff *et al.*, 2001; Taniguchi *et al.*, 2002). The expression of NO synthase II is correlated with cytotoxic or cytostatic events and results in a sustained synthesis of NO, which in turn induces apoptotic cell death (Rosselli *et al.*, 1998). These findings confirmed our suggestion that less evident changes in progesterone production observed after ($\pm$)-2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine treatment may be a result of less NO synthase II activity in the bovine mid-luteal cells.

In the present study, treatment with NO synthase inhibitors decreased NO production and increased secretion of progesterone. These findings differ in part from the results of Olson *et al.* (1996), which indicated that treatment with N^{ω} -nitro-L-arginine methyl ester reduced production of endogenous NO in a dose-dependent manner but did not affect progesterone production by cells obtained from luteinized rat ovaries. However, the stimulatory influence of NO synthase inhibitors on progesterone secretion observed in the present study is in agreement with the results obtained after addition of N^{ω} -nitro-L-arginine methyl ester or N^G -methyl-L-arginine to incubated corpora lutea of pseudopregnant rats (Motta and Gimeno, 1997) and rabbits (Gobbetti *et al.*, 1999).

Other studies indicate that NO plays a role not only as a modulator of steroidogenesis but also as survival or cell death induction factor in the ovary (Basini *et al.*, 1998). The dual effects of NO seem to be dose-dependent (Basini *et al.*, 1998) and cell type-specific (Burney *et al.*, 1997). High concentration of NO inhibits DNA fragmentation in the bovine granulosa cells and in low concentration stimulates cellular apoptosis in granulosa cells only from large follicles (Basini *et al.*, 1998). Recently it was shown that spermine-NO complex strongly reduced viability of the bovine luteal cells, whereas N^{ω} -nitro-L-arginine methyl ester indicates its opposite effect (A. Korzekwa, K. Okuda, J. J. Jaroszewski and D. J. Skarzynski, unpublished). Therefore, inhibition of progesterone production after treatment with NO donors, observed in the present study, may be a consequence of inhibition of steroidogenesis and reduction of the viability of luteal cells.

NO is known to inhibit progesterone production directly via inhibition of enzymes involved in steroidogenesis (Wink *et al.*, 1993) or indirectly via activation of some luteolytic factors, that is endothelin 1 (Levy *et al.*, 2000; Hinckley and Milvae, 2001) or leukotriene C_4 (Bogacki *et al.* 2002). Therefore, blocking NO production by NO synthase inhibitors associated with increase of progesterone secretion, observed in bovine corpora lutea, may also be a result of abolition of the NO inhibitory direct effect on steroidogenic enzymes or indirect luteolytic factors output. The NO-NO synthase system-related luteotrophic or luteolytic effects triggered in rabbit corpora lutea by exogenous PGE_2 or $PGF_{2\alpha}$ *in vitro* were observed by Boiti *et al.* (2000). During simultaneous administration of N^{ω} -nitro-L-arginine methyl ester and dinoprost (an analogue of $PGF_{2\alpha}$) into the bovine corpora lutea *in vivo*, N^{ω} -nitro-L-arginine methyl ester inhibited secretion of luteolytic $PGF_{2\alpha}$ but not luteotrophic PGE_2 (Bogacki *et al.*, 2002). This finding confirmed that the stimulation of progesterone production by NO synthase inhibitor may be in part mediated indirectly by the changes in arachidonic acid pathway. Therefore, NO appears to play an important role in the regulation of corpus luteum function as one of many components of an autocrine or paracrine cascade; however, a complete explanation of its role still needs further study.

In conclusion, the results of the present study demonstrate that nitric oxide donors inhibit and nitric oxide inhibitors increase progesterone production, by cultured bovine luteal cells at days 10–14 of the oestrous cycle. The degree of progesterone inhibition after NO donor treatment and stimulation after NO synthase inhibitors is dependent on the donor or inhibitor used. The results of the present study resolve some of the previous conflicting reports, since they compare the donors and inhibitors under identical conditions.

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References

- Basini G, Baratta M, Ponderato N, Bussolati S and Tamanini C (1998) Is nitric oxide an autocrine modulator of bovine granulosa cell function? *Reproduction, Fertility and Development* **10** 471–478
- Bogacki M, Jaroszewski JJ, Skarzynski DJ and Hansel W (2002) The influence of nitric oxide on cyclooxygenase and lipoxygenase products of arachidonic acid metabolism in the bovine corpus luteum *in vivo*. *Biology of Reproduction* **66** Supplement 1 325 (Abstract)
- Bogdan C (2001) Nitric oxide and the immune response *Nature Immunology* **2** 907–916
- Boiti C, Zerani M, Zampini D and Gobbetti A (2000) Nitric oxide synthase activity and progesterone release by isolated corpora lutea of rabbits in the early and mid-luteal phases of pseudopregnancy are modulated differentially by prostaglandin E_2 and prostaglandin $F_{2\alpha}$ via adenylyl cyclase and phospholipase C *Journal of Endocrinology* **164** 179–186
- Burney S, Tamir S, Gal A and Tannenbaum SR (1997) A mechanistic analysis of nitric oxide-induced cellular toxicity *Nitric Oxide* **1** 130–144
- Dong YL, Gangula PRR and Yallampalli C (1996) Nitric oxide synthase isoforms in the rat uterus: differential regulation during pregnancy and labour *Journal of Reproduction and Fertility* **107** 249–254
- Dong YL, Gangula PRR, Fang L and Yallampalli C (1999) Nitric oxide reverses prostaglandin-induced inhibition in ovarian progesterone secretion in rats *Human Reproduction* **14** 27–32
- Drazen DL, Klein SL, Burnett AL, Wallach EE, Crone JK, Huang PL and Nelson RJ (1999) Reproductive function in female mice lacking the gene for endothelial nitric oxide synthase *Nitric Oxide* **3** 366–374
- Dunnam RC, Hill MJ, Lawson DM and Dunbar JC (1999) Ovarian hormone secretory response to gonadotropins and nitric oxide following chronic nitric oxide deficiency in the rat *Biology of Reproduction* **60** 959–963
- Eisenstein TK (2001) Implications of Salmonella-induced nitric oxide (NO) for host defense and vaccines: NO, an antimicrobial, antitumor immunosuppressive and immunoregulatory molecule *Microbes and Infection* **3** 1223–1231
- Friden BE, Runesson E, Hahlin M and Brannstrom M (2000) Evidence for nitric oxide acting as a luteolytic factor in the human corpus luteum *Molecular Human Reproduction* **6** 397–403
- Gobbetti A, Boiti C, Canali C and Zerani M (1999) Nitric oxide synthase acutely regulates progesterone production by *in vitro* cultured rabbit corpora lutea *Journal of Endocrinology* **160** 275–283
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR (1982) Analysis of nitrate, nitrite and ^{15}N nitrate in biological fluids *Analytical Biochemistry* **126** 131–138
- Hinckley ST and Milvae RA (2001) Endothelin-1 mediates prostaglandin $F_{2\alpha}$ -induced luteal regression in the ewe *Biology of Reproduction* **64** 1619–1623
- Ignarro LJ (1990) Biosynthesis and metabolism of endothelium-derived nitric oxide *Annual Review of Pharmacology and Toxicology* **30** 535–560
- Jablonka-Shariff A and Olson LM (1998) The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knock-out mouse oocytes *Endocrinology* **139** 2944–2954
- Jaroszewski JJ and Hansel W (2000) Intraluteal administration of a nitric oxide synthase blocker stimulates progesterone and oxytocin secretion and prolongs the life span of the bovine corpus luteum *Proceedings of the Society for Experimental Biology and Medicine* **224** 50–55
- Jaroszewski JJ, Skarzynski DJ and Okuda K (2001) Nitric oxide as a local regulator in the mammalian ovary. In *Reproductive Biotechnology: Reproductive Biotechnology Update and Its Related Physiology* pp 105–112 Eds H Miyamoto and N Manabe. Elsevier, Tokyo
- Kiss JP (2000) Role of nitric oxide in the regulation of monoaminergic neurotransmission *Brain Research Bulletin* **52** 459–466

- Levy N, Kobayashi S, Roth Z, Wolfenson D, Miyamoto A and Meidan R (2000) Administration of prostaglandin $F_{2\alpha}$ during the early bovine luteal phase does not alter the expression of ET-1 and of its type A receptor: a possible cause for corpus luteum refractoriness *Biology of Reproduction* **63** 377–382
- Miyamoto Y, Skarzynski DJ and Okuda K (2000) Is tumor necrosis factor- α a trigger for the initiation of endometrial prostaglandin $F_{2\alpha}$ release at luteolysis in cattle? *Biology of Reproduction* **62** 1109–1115
- Moncada S, Palmer RMJ and Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology *Pharmacological Reviews* **43** 109–142
- Motta AB and Gimeno MAF (1997) Nitric oxide participates in the corpus luteum regression in ovaries isolated from pseudopregnant rats *Canadian Journal of Physiology and Pharmacology* **75** 1335–1339
- Motta AB, Estevez A, Tognetti T, Gimeno MAF and Franchi AM (2001) Dual effects of nitric oxide in functional and regressing rat corpus luteum *Molecular Human Reproduction* **7** 43–47
- Okuda K, Uenoyama Y, Fujita Y, Iga K, Sakamoto K and Kimura T (1997) Functional oxytocin receptors in bovine granulosa cells *Biology of Reproduction* **56** 625–631
- Olson LM, Jones-Burton CM and Jablonka-Shariff A (1996) Nitric oxide decreases estradiol synthesis of rat luteinized ovarian cells: possible role for nitric oxide in functional luteal regression *Endocrinology* **137** 3531–3539
- Pate JL and Landis Keyes P (2001) Immune cells in the corpus luteum: friends or foes *Reproduction* **122** 665–676
- Petroff MG, Petroff BK and Pate JL (2001) Mechanisms of cytokine-induced death of cultured bovine luteal cells *Reproduction* **121** 753–760
- Rosselli M, Keller PJ and Dubey RK (1998) Role of nitric oxide in the biology, physiology and pathophysiology of reproduction *Human Reproduction Update* **4** 3–24
- Sessa WC (1994) The nitric oxide synthase family of proteins *Journal of Vascular Research* **31** 131–143
- Skarzynski DJ and Okuda K (1999) Sensitivity of bovine corpora lutea to prostaglandin $F_{2\alpha}$ is dependent on progesterone, oxytocin and prostaglandins *Biology of Reproduction* **60** 1292–1298
- Skarzynski DJ and Okuda K (2000) Different actions of noradrenaline and nitric oxide on the output of prostaglandins and progesterone in cultured bovine luteal cells *Prostaglandins and Other Lipid Mediators* **60** 35–47
- Skarzynski DJ, Kobayashi S and Okuda K (2000) Influence of nitric oxide and noradrenaline on prostaglandin $PGF_{2\alpha}$ -induced oxytocin secretion and intracellular calcium mobilization in cultured bovine luteal cells *Biology of Reproduction* **63** 1000–1005
- Skarzynski DJ, Jaroszewski JJ, Bah MM, Deptula KM, Barszczewska B and Gawronska B (2001) An inhibitor of nitric oxide synthase (L-NAME) blocks prostaglandin $PGF_{2\alpha}$ -induced luteolysis in cattle *Biotechnology, Agronomy Society and Environment* **5** 48 (P28) (Abstract)
- Taniguchi H, Yokomizo Y and Okuda K (2002) Fas–Fas ligand system mediates luteal cell death in bovine corpus luteum *Biology of Reproduction* **66** 754–759
- Van Voorhis BJ, Dunn MS, Snyder GD and Weiner CP (1994) Nitric oxide: an autocrine regulator of human granulosa–luteal cell steroidogenesis *Endocrinology* **135** 1799–1806
- Wink DA, Osawa Y, Darbyshire JF, Jones CR, Eshenaur SC and Nims RW (1993) Inhibition of cytochromes progesterone 50 by nitric oxide and a nitric oxide-releasing agent *Archives of Biochemistry and Biophysics* **300** 115–123

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