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Antisteroidogenic Action of Nitric Oxide on Human Corpus Luteum In Vitro

Mechanism of Action

M. Cecilia Johnson,[1] Hugo A. Diaz,[1,2] Carlos Stocco,[1,3] Alberto Palomino,[1,4] Luigi Devoto,[1] and Margarita Vega[1]

[1]Institute of Maternal and Child Research, School of Medicine, University of Chile. Santiago, Chile; [2]Department of Biology, School of Science, University of Valparaiso, Valparaiso, Chile; [3]Postdoctoral Fellow (PLACIRH), School of Medicine, University of Chile; and [4]Reproductive Medicine and Infertility, School of Medicine, University of Chile

To analyze the mechanism by which nitric oxide (NO) exerts its antisteroidogenic action, human luteal cells were cultured during 24 and 48 h with L-arginine (L-Arg, 1 mmol/L); 1,2(2-trifluoromethylphenyl)imidazole (TRIM) (50 μmol/L and 1 mmol/L) and cyclic guanosine monophosphate (cGMP) analog (8-Br-cGMP, 1 mmol/L). Estradiol, nitrite, and $P_{450\ AROM}$ activity were determined in culture media. Total cGMP concentration was evaluated in the cells and culture media by radioimmunoassay, and NADPH diaphorase was used as a histochemical marker for NO synthase (NOS) activity. During the corpus luteum (CL) life-span, NO affected estradiol secretion in an age-dependent manner, with an inhibition in mid-CL (37%; $p < 0.05$) in agreement with our previous results, and no significant modification in early and late CL. Basal nitrite concentration in 24 and 48 h of midluteal cell cultures (42 and 93 pmol/10^6 cells, respectively) was increased by L-Arg (53% and 88%) and inhibited by the two TRIM concentrations; also, an intense diaphorase reactivity was observed in endothelial cells and luteal parenchyma. Total cGMP was not detected in cell cultures and 8-Br-cGMP did not modify estradiol secretion, whereas aromatase activity was strongly inhibited by L-Arg (70%, $p < .05$). These results suggest that both NOS isoforms are active in midluteal cells, and the mechanism of action for NO on in vitro estradiol secretion may be an inhibition of $P_{450\ AROM}$ activity.

Key words: nitric oxide; nitric oxide synthase; $P_{450\ AROM}$; NADPH diaphorase.

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Author to whom all correspondence and reprint requests should be addressed: Dr. Margarita Vega, Institute of Maternal and Child Research, University of Medicine, School of Medicine, P.O.Box 226–3, Santiago, Chile. Email: mvega@machi.med.uchile.cl

Introduction

The corpus luteum (CL) is a transient gland composed by different cell types, such as, large and small steroidogenic cells, fibroblasts, endothelial cells, and leukocytes *(1,2)*. The percentage of each cell type varies throughout the luteal phase, and the secretion of diverse substances by each cell subpopulation contributes to the development, maturity, and regression of the CL. In addition, these multiple products, including growth factors, cytokines, and reactive oxygen species, may be responsible for the modulation of steroid synthesis in mammalian corpora lutea *(2–5)*.

Recent studies have shown that nitric oxide (NO) is a novel regulator of different physiological functions, some of which are associated with the reproductive process, including mammalian ovulation, follicle maturation, luteal regression, and granulosa, luteal, and Leydig cell steroidogenesis *(5–10)*. This short half-life gas is generated from L-arginine (L-Arg) by the action of constitutive (calcium-dependent) or inducible (calcium-independent) isoforms of nitric oxide synthase (NOS). Two constitutive isoforms of NOS were first identified in brain (nNOS) and in endothelium (eNOS), and the inducible isoform (iNOS) was isolated from macrophages *(11–14)*. Recently, we have shown the expression of eNOS mRNA in human CL during its life-span, being highest in CL obtained in the midluteal phase *(9)*. Moreover, we have detected both isoenzyme proteins, eNOS and iNOS, in the luteal parenchyma with a greater staining for the eNOS isoform. On the other hand, it has been shown that NO elicits a negative effect on the in vitro midluteal steroidogenesis, mainly on estradiol (E_2) production *(9)*, in agreement with the data reported by Olson et al. *(6)* and Van Voorhis et al. *(7)*.

The gaseous nature of NO raises the possibility of a direct interaction of this free radical with intracellular systems of adjacent cells. In this regard, it has been reported that NO exerts many of its functions by binding to the heme-containing

proteins, such as guanylate cyclase with cyclic GMP (cGMP) formation, cyclooxygenase, and cytochrome P_{450} steroidogenic enzymes, inducing either an activation or inhibition of the target enzyme *(11–15)*.

The aim of the present research was to further analyze the participation of the NO–NOS system on human CL lifespan and its mechanism of action on the in vitro human luteal E_2 production.

Results

The effect of L-Arg, NOS substrate, on E_2 secretion was determined in cultures of human luteal cells obtained from early, mid, and late CL, in the presence of L-Arg, the NO substrate (Fig. 1). The results show a significant inhibitory effect of NO on E_2 production by cells obtained from mid CL, confirming our previous findings *(9)*. In fact, L-Arg, inhibited E_2 secretion by 37% compared to basal values, ($p < 0.05$). Similar results were obtained in cells incubated with sodium nitroprusside, NO-generating drug (data not shown). In contrast, E_2 secretion by early or late luteal cells in the presence of NO generating substances, was not significantly affected. Based on these data, the following experiments were performed in cells obtained from mid-CL.

The action of 1,2(2-trifluoromethylphenyl)imidazole (TRIM), a potent inhibitor of nNOS and iNOS (IC_{50} of 28.2 and 27.0 μmol/L, respectively), and a relatively weak inhibitor of eNOS (IC_{50} 1057.5 μmol/L) *(16)*, on E_2 production by mid-cell cultures was studied; 50 μmol/L TRIM increased E_2 secretion by 88% (basal values: 352 ± 48 pg/10^6 cells, TRIM 50 μmol/L: 664 ± 128 pg/10^6 cells), $p < 0.05$.

The activity of NOS was evaluated by studying NADPH diaphorase activity, and by nitrite production during culture. Based on the reported colocalization of the activities of NADPH diaphorase and NOS, this assay was used as a histochemical marker for the activity of all the isoforms of NOS present in the CL *(11,14,17)* (Fig. 2). The reaction produces a dark blue precipitate, which was more intense at the periphery of the parenchyma (Fig. 2A). The positive staining was also observed at the endothelium of blood vessels and decreased toward the central cavity, where no reactivity was observed.

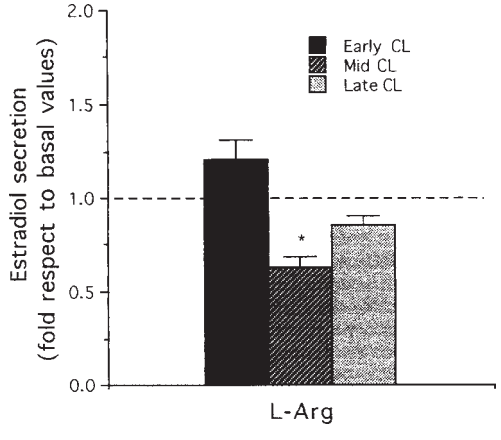

Fig. 1. Effect of NO donor, L-Arg, on E_2 secretion by early, mid, and late luteal cell cultures. Precultured luteal cells obtained from different stages of the luteal phase were incubated for 24 h in the presence of L-Arg (1 mmol/L). E_2 secretion was measured as described in Material and Methods. Results are the mean ± SEM of five CL from each stage and are presented as fold of basal values. Estradiol basal values for early, mid and late luteal cells were 354.0 ± 72; 502.0 ± 46.1 and 792.0 ± 326.0 pg/10^6 cells, respectively. *$p < 0.05$ vs basal values.

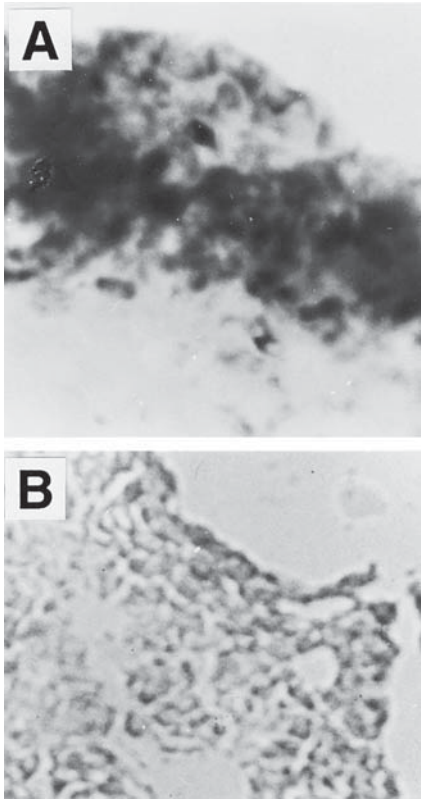

Fig. 2. Histochemical study of NADPH diaphorase activity in mid CL. **(A)** Positive staining, which is evidenced by a dark blue precipitate, was detected at the peripheral parenchyma of cryosection of human CL. **(B)** Control negative was performed in the absence of NADPH, as described in the Material and Methods section. Original magnification ×400.

The nitrite concentration was determined in the culture media of mid luteal cells (Fig. 3). A twofold increase in basal nitrite concentration was observed at 48 h of culture compared to the 24-h culture (92.8 ± 2.4 nmol/10^6 cells vs 42.5 ± 4.5 nmol/10^6 cells, respectively, $p < 0.05$). When L-Arg was added to the media, an increase in nitrite concentration of 53 ± 6% and 88 ± 3% was obtained in cells cultured for 24 and 48 h, respectively. By contrast, the incubation of the cells with 50 μmol/L or 1 mmol/L TRIM reduced nitrite production during the culture period (50 μmol/L: 65 ± 4% and 34 ± 6%; 1 mmol/L: 56 ± 7% and 32 ± 5% vs basal values, for 24- and 48-h cultures, respectively, $p < 0.05$).

The negative action of NO on E_2 secretion may be mediated by the activation of guanylyl cyclase and generation of

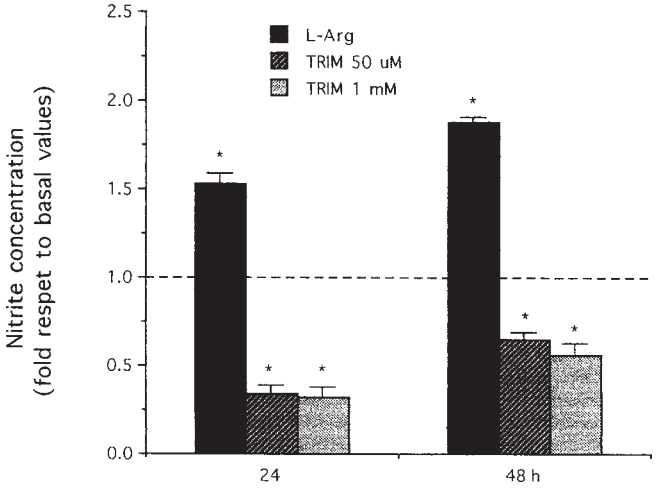

Fig. 3. Nitrite concentration in midluteal cell cultures. Precultured midluteal cells were treated for 24 or 48 h in the presence of L-Arg (1 mmol/L) or TRIM (50 μmol/L or 1 mmol/L). Nitrite concentration was quantitated in the culture media as described in the Material and Methods section. Results are the mean ± SEM of four separate experiments performed in duplicate and are present as fold of basal values. Basal values: 24 h = 42.5 ± 4.5; and 48 h = 92.8 ± 2.4 nmol/10^6 cells. *$p < 0.05$ vs basal values.

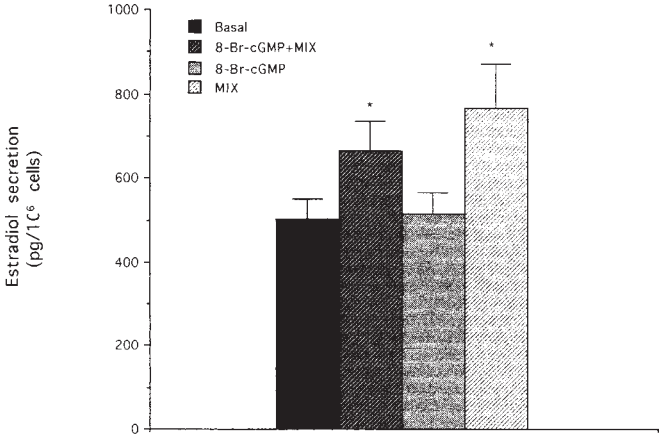

Fig. 4. Action of the cGMP analog on E_2 secretion in mid luteal cell cultures. Precultured mid luteal cells were incubated with 8-Br-cGMP (1 mmol/L) in the presence and in the absence of IBMX (0.1 mmol/L) in the same experiments. Estradiol secretion was measured in the culture media by RIA. Results are the mean ± SEM of four separate experiments performed in duplicate. *$p < 0.05$ vs basal values.

cGMP in midluteal cell culture. Total cGMP concentration was not detectable, even in the presence of L-Arg. In parallel studies, the effect of 8-Br-cGMP on E_2 secretion was analyzed in the presence and in the absence of the phosphodiesterase inhibitor, isobutylmethyl-xanthine (IBMX) (Fig. 4). The addition of the cGMP analog and IBMX increases E_2 production when compared to basal values, in 46% ($p < 0.05$). To elucidate whether this stimulatory effect was by the cGMP analog or by the action of IBMX, luteal cells were cultured with each compound separately in the same experiments. E_2 production was unchanged in the presence of the cGMP analog, whereas, IBMX stimulated E_2 secretion in 53%, ($p < 0.05$), suggesting that the positive effect observed on steroid synthesis with the combination of 8-Br-cGMP and IBMX, may be due to the action of the phosphodiesterase inhibitor rather than to the cGMP analog itself.

The other possible mechanism of action by which NO may be involved in the regulation of luteal E_2 production, is on the activity of $P_{450\ AROM}$ (Fig. 5). Mid luteal cells treated with L-Arg were incubated in the presence of testosterone (T) by additional 3 h. The E_2 secretion in the presence of an excess of T was 205 ± 45 pg/10^6 cells (control 0 h: 516 ± 85 pg/10^6 cells; basal 3 h: 721 ± 92 pg/10^6 cells), indicating the capacity of cultured luteal cells to synthetize E_2. Nevertheless, in cells previously treated with L-Arg, E_2 production diminished by 71% vs basal values ($p < 0.05$), suggestive of a decrease on $P_{450\ AROM}$ activity.

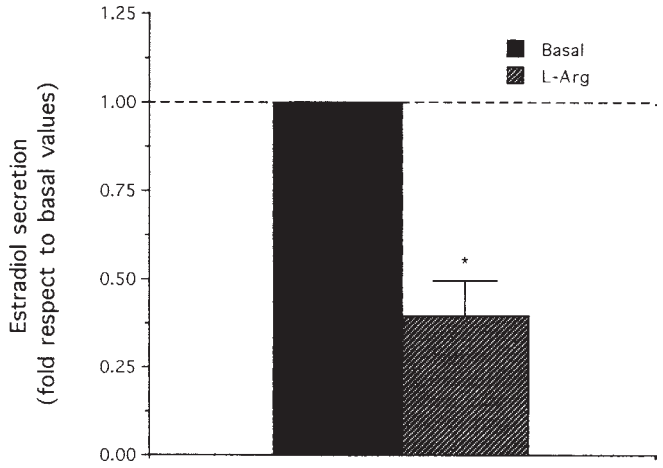

Fig. 5. Action of NO on $P_{450\ AROM}$ activity. Precultured mid luteal cells were treated with L-Arg (1 mmol/L) for 24 h. The medium was replaced and an excess of T (1 μmol/L) was added to the plates. After 3 h, the media were separated and E_2 concentration was determined. Results are the mean ± SEM of four separate experiments and are presented as fold of basal values. $Control_0$ values correspond to the E_2 concentration found in the culture media after the addition of T and previous to the 3h-incubation. $Control_0$ values= 516 ± 85 pg/10^6 cells; $Basal_{3h}$ values = 721 ± 92 pg/10^6 cells. *$p < 0.05$ vs basal values.

Discussion

Nitric oxide has emerged as an important autocrine-paracrine regulator of several intraovarian functions. Recently, our group reported that the NO/NOS system has an antisteroidogenic in vitro action on luteal cells, particularly on E_2 production *(9)*. In this regard, the data of the present study further established that E_2 secretion by cells from early and late CL, was not importantly modified by

the action of NO. This age-dependency effect of NO on E_2 secretion may be partially explained by the predominance of certain cell types in each stage of the CL and, therefore, the existence of different microenviroments within the luteal tissue. It is well accepted that the human CL is heterogeneous in its cell composition, including endothelial cells, fibroblasts and leukocytes, besides the steroidogenic small and large luteal cells, whose proportion changes during the life-span of the CL *(1,18,19)*.

It has been reported that eNOS responds to stimuli by producing small quantities of NO, whereas the stimulation of iNOS results in new protein synthesis and a substained production of NO over long periods *(12)*. In this regard, both isoforms, iNOS and eNOS, have been immunodetected in human luteal parenchyma, eNOS being the more abundant isoform present *(9)*. Furthermore, in this investigation the staining pattern found for NADPH diaphorase, which is suggestive of NOS activity, was in agreement with our previous immunohistochemical study. In addition, the activity of NOS was clearly evidenced by the large amount of nitrites found in human midluteal cell cultures. Similar results were observed in rat luteinized ovarian cells *(6)*. Even though this study was not performed in the presence of an inhibitor of protein synthesis, the decrease in nitrite poduction with the selective NOS inhibitor, supports the proposition that iNOS may be the isoform that mainly generates NO in midluteal cells. In the cell culture system used in this study, we have observed that several cytokines that may induced iNOS, including interleukin-1β (IL-1β) *(1)*, tumor necrosis factor α (TNF-α) and interferon-gamma are secreted by the different luteal cell types (unpublished data). Nevertheless, the large number of cells expressing eNOS in mid-human CL *(9)*, besides the amount of nitrites found in the presence of TRIM, may contribute to the constant production of NO in the luteal tissue.

It is well known that NO is an endothelial-derived relaxing factor, action mediated by the binding of NO to the iron of the heme at the active site of guanylyl cyclase, inducing cGMP synthesis *(11)*. In human granulosa-luteal cells, NO increases cGMP concentration in the presence or absence of the phosphosdiesterase inhibitor *(7)*. In addition, in immature rat ovarian cells and bovine thecal cells in vitro, the NO-cGMP signal transduction pathway was activated by IL-1β and TNF-α *(20,21)*. However, in bovine granulosa cells, the cGMP concentration induced by NO was below the sensitivity of the assay *(20)*. Similarly, our results showed that total cGMP was not detectable in human luteal cell cultures; nevertheless, we cannot rule out the possibility of cGMP hydrolysis by phosphodiesterases present in the human CL. Moreover, the cGMP analog, 8-Br-cGMP, had no effect on human luteal steroidogenesis, in agreement with the data reported for human granulosa-luteal cells *(7)*. It is most likely that the increase in E_2 secretion observed in our study in the presence of the phosphodiesterase inhibitor, may reside in the accumulation of cAMP, which is a well-known stimulator of human luteal steroidogenesis *(4)*. By contrast, La Polt and Hong *(22)* have reported a decrease on E_2 production induced by the analog $(Bu)_2$cGMP in rat granulosa cells. These contradictory results may be due to the different experimental conditions used in both studies, such as species, cGMP analogs, and cell types. The data presented in this study strongly suggest that the inhibitory effect of NO on steroidogenesis is not mediated by the activation of guanylyl cyclase and cGMP generation.

Another possible mechanism for the antisterodogenic action of NO may be explained by the modulation of the activity of cytochrome P_{450} steroidogenic enzymes. The decrease in the levels of E_2 biosynthesis observed in the present study, are closely related to the changes reported for the aromatase transcription in human follicles and corpora lutea throughout the menstrual cycle *(23,24)*. However, aromatase activity is inhibited in cultured granulosa-luteal cells by the action of NO when they are exposed for only 1 h, indicating a direct effect of this free radical on the enzyme activity, rather than on new mRNA synthesis *(15)*. In addition to the action of NO on the iron of the heme group, recently it has been proposed that NO may bind to the sulfhydryl group of the cysteine residues located in the active site of all P_{450} steroidogenic enzymes with the formation of nitrosothiol group and the concomitant inhibition of the enzymatic activity *(15,25)*.

In summary, the present study provides evidences of the activity of both NOS isoforms and that NO exerts an age-dependent antisteroidogenic effect on human cultured luteal cells, affecting $P_{450\,AROM}$ activity, independent of the cGMP pathway. These results are in agreement with our previous studies *(9)*, showing the expression and the presence of NOS in the human CL and the NO-induced decrease in steroid secretion. Undoubtedly, the cell-to-cell interaction, represented by the diversity of cell types within the CL with the capacity to produce several compounds including NO, constitutes an attractive model of autocrine and/or paracrine regulation of the events in which the CL is involved.

Material and Methods

Subjects

Corpora lutea were obtained from 20 normal women requesting surgical sterilization at the San Borja-Arriarán Clinical Hospital. This procedure was approved by the Institutional Review Board of the Hospital and informed written consent was obtained from all patients before surgery. Plasma levels of progesterone (P) and histologic analysis of endometrial and CL-dating biopsies, showed that the CL corresponded to different stages of the luteal phase: early (<5 d after LH peak, P = 4.2 ± 0.7 ng/mL, n = 5), mid (5–9 d after LH peak, P = 7.5 ± 0.6 ng/mL, n = 10) and late (<9 d after LH peak, P = 4.6 ± 0.7 ng/mL, n = 5). After

removal, the tissue was placed in sterile 0.15 mmol/L NaCl and transported to the laboratory at room temperature.

Cell Dispersion and Culture

Human CL were dispersed as previously described with some modifications *(26)*. Briefly, luteal tissue was minced, washed, and digested for 90 min with collagenase (370 U/100 mg tissue, Worthington Biochemical Corp. Freehold, NJ) and DNAse I (14 KU/100mg tissue, Sigma Chemical Co., St Louis, MO) in medium 199 (M199) containing $NaHCO_3$ (26 mmo/L), BSA (0.1% w/v), HEPES (25 mmol/L), antibiotics (100 IU/mL penicillin and 5 mg/mL streptomycin). Then, luteal cells were separated from red blood cells by a Ficoll solution (hystopaque, α = 1.077 g/mL), and washed twice with phosphate-buffered saline (PBS, pH7.4)-BSA 1%. Cells were counted in an hemocytometer and the viability obtained was >85% and was unchanged during the time culture and in the presence of different agents, as assessed by the trypan blue exclusion method. Aliquots of 10^5 cells were plated in 1 mL of defined M199 at 37°C in a 5% CO_2-air atmosphere. After 24 h, adherent cells were washed and M199 was replaced by Hank's (L-Arg-free) supplemented with glutamine (0.1 mg/mL), BSA, $NaHCO_3$, HEPES, and antibiotics. The cells (8–9 × 10^4 cells/plate) were cultured for another 24 h in the absence (basal condition) and in the presence of NOS substrate, L-Arg (1 mmol/L, Sigma); TRIM (50 μmol/L, iNOS inhibitor and 1 mmol/L, weak inhibitor of eNOS activity *(16)*, Calbiochem-Novabiochem); 8-Br-cGMP (a membrane-permeable, nonhydrolyzable analog cGMP, 1 mmol/L, Sigma) with and without isobutyl-methyl-xanthine (IBMX, 0.1 mmol/L, Sigma). The cell incubation was terminated as indicated in each study.

Hormone Assay

The culture media were separated and stored at –20°C until assayed for E_2 by specific RIA, as described previously *(26)*. The cross-reaction of the antiserum was <0.02%, 0.8%, and <0.02% with testosterone, estriol and estrone, respectively.

Determination of $P_{450\ AROM}$ Activity

Based on the great capacity of human midluteal cells to synthesize E_2, $P_{450\ AROM}$ activity was assessed by studying the conversion of androgens to estrogens. Briefly, after 24 h incubation in Hank's in the presence of L-Arg, the medium was replaced and an excess of T (1 μmol/L, Sigma) was added to some plates. After 3 h, the media were separated and frozen at –20°C until assayed for E_2, which was identified by thin layer chromatography.

Determination of cGMP Concentration

Total cGMP concentration was measured in cell homogenates and culture media by RIA, as described previously *(27)*. Briefly, after incubation, the cells and media were frozen and thawed twice, boiled for 10 min, centrifuged and freeze-dried. The samples were resuspended in 0.05 mol/L sodium acetate buffer (pH 6.5) and cGMP was measured by RIA after acetylation by the mixture triethanolamine:acetic anhydride (1:2, v/v). An overnight incubation at 4°C in the presence of ^{125}I-2'-*O*-succinyl-cGMP-tyrosyl-methyl-ester (S-cGMP-TMP) and polyclonal antibody in a final dilution of 1:40.000, was performed. Bound radioactivity was separated by precipitation with ammonium sulfate *(50%)* in the presence of 200 μg IgG, and measured in a gamma counter spectrometer LKB Wallac. The sensitivity of the RIA was 20 fmol/mL.

Determination of NOS Activity

Nitrite Concentration

Nitric oxide synthase activity was determined by measuring nitrite concentration, as described by Samlowski et al. *(28)* with modifications. Luteal cells obtained from mid CL were cultured in Hank's media for either 24 or 48 h with L-Arg (1 mmol/L) or TRIM (50 μmol/L or 1 mmol/L). Then, 200 μL culture media were incubated with 200 μL Griess Reagent [1:1 mixture (v/v) 0.6% sulfanilic acid in HCl 3 mol/L:0.4% *N*-(1-naphtyl)ethylenediamine diHCl in HCl 3 mol/L]. The absorbance at 540 nm was measured in a spectrophotometer Perkin-Elmer Coleman 55 (Perkin Elmer Co. Oak Brook, IL). Concentrations were determined from a linear standard curve generated from 0.75 and 100 μmol/L sodium nitrite in medium.

NADPH Diaphorase Activity

Frozen sections (10 μm) were obtained from mid CL and fixed in 4% formaldehyde/PBS for 16–18 h at 4°C, followed by 10% sucrose cryoprotection. After washing in PBS, staining for NADPH diaphorase activity was assessed by incubating the sections in 50 mmol/L Tris-HCl, pH 8.0 containing 1 mmol/L β-NADPH, 0.5 mmol/L nitroblue tetrazolium and 0.25% Triton X-100, for 30 min at 37°C. Control sections were performed in the absence of NADPH. The dark blue NADPH diaphorase positive staining was observed in a Nikon optical microcospe (Nikon Inc., Melville, NY).

Statistical Analysis

The results were normalized to 10^6 viable cells. The data are presented as mean ± SEM for the number of separate studies as indicated in the figure legends. The results were analyzed using Student's *t*-test for unpaired results, or one-way analysis of variance, using Fisher's protected least-squares difference multiple-comparison test. $p < 0.05$ was considered statistically significant.

Acknowledgments

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