Endocrine, *vol. 8, no. 2, 185–191, April 1998* 0969–711X/98/8:185–191/$9.75 © 1998 by Humana Press Inc. All rights of any nature whatsoever reserved.

Regulation of Human Luteal Steroidogenesis In Vitro by Nitric Oxide

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To evaluate the effect of nitric oxide (NO·) in human corpus luteum (CL) function, we investigated the expression and the presence of NO· synthase (NOS) in the human CL and the action of NO· on the in vitro luteal steroid production. The expression of endothelial NOS (eNOS) in early, mid-, and late CL was assessed by reverse transcriptase polymerase chain reaction (RT-PCR) and the immunohistochemical study was performed in human CL histological sections by using monoclonal antibodies (MAbs) against the distinct NOS isoforms. In addition, seven human mid-CLs were enzymatically dispersed, and the cells were cultured with NO· donor compounds. Steroid production was measured in the culture media by specific radioimmunoassay. The results show that the expression of eNOS was highly detected in mid- and early CL, and to a lesser extent, in late CL. Meanwhile, the immunohistochemical study indicated that both isoenzymes of NOS were expressed in mid-human CL, eNOS being the more abundant isoform present. On the other hand, functional studies showed that NO· donors (L-arginine [L-Arg] and sodium nitroprusside) elicited an inhibitory action on steroid synthesis, preferentially on estradiol production by the luteal cell cultures ($p < 0.05$). In conclusion, the NO·–NOS system is present in the human CL, and it may serve as a modulator of the in vitro human luteal steroidogenesis.

Key Words: Steroidogenesis; nitric oxide; nitric oxide synthase; human corpus luteum.

Introduction

Several studies have provided convincing evidence that reactive oxygen species (ROS) generated in the mammalian ovary *(1,2)* act as important modulators of the function of this organ. We have previously shown that an oxidative stress condition is established in the mid stage human corpus luteum (CL), suggesting an important role of ROS in human CL involution *(3)*. Moreover, the existence of leukocytes and endothelial cells as normal cellular elements of the CL suggests the possibility of intraluteal generation of ROS that can modulate human luteal steroidogenesis *(3–5)*.

Recent studies have established that a short-lived gas with free radical chemical properties, nitric oxide (NO·), plays a major role in endocrine regulation *(6)*, in addition to its involvement in other physiological events *(7–9)*. It has been shown that NO· is generated in mammalian cells from L-arginine (L-Arg) by the action of multiple isoenzymes of NO· synthase (NOS). The release of large amounts of NO· is characteristic for induction of inducible or macrophage NOS (macNOS), whereas the endothelial NOS (eNOS) isoform produces small amounts of NO· *(10–12)*. This free radical serves as a biological messenger molecule by acting as an intra- and intercellular signaling mechanism *(8)*. NO· might exert many of its physiological actions by binding to the iron of the heme-containing enzymes, such as guanylyl cyclase *(13,14)*, cyclooxygenase *(15)*, and cytochrome P_{450} steroidogenic enzymes *(16)*. In fact, a recent investigation indicated that NO· may act as an autocrine regulator of steroid production in human granulosa cells obtained from women undergoing in vitro fertilization procedures *(16)*. However, there is no evidence about the participation of NO· as a regulatory molecule of steroid secretion by human luteal cells, although the cellular composition of the human CL suggests the possibility of intraluteal production of NO·. The present study was designed to examine the potential role of the system NO·–NOS on human luteal steroidogenesis.

Results

The presence of specific eNOS mRNA was detected in human CL of different ages, as observed in a representative gel shown in Fig. 1. The resulting 485-, 290-, and 120-bp amplification products were the size predicted based on the cDNA sequence of NOS, abl, and β_2-microglobulin,

Received October 31, 1997; Revised January 20, 1998; Accepted January 20, 1998.

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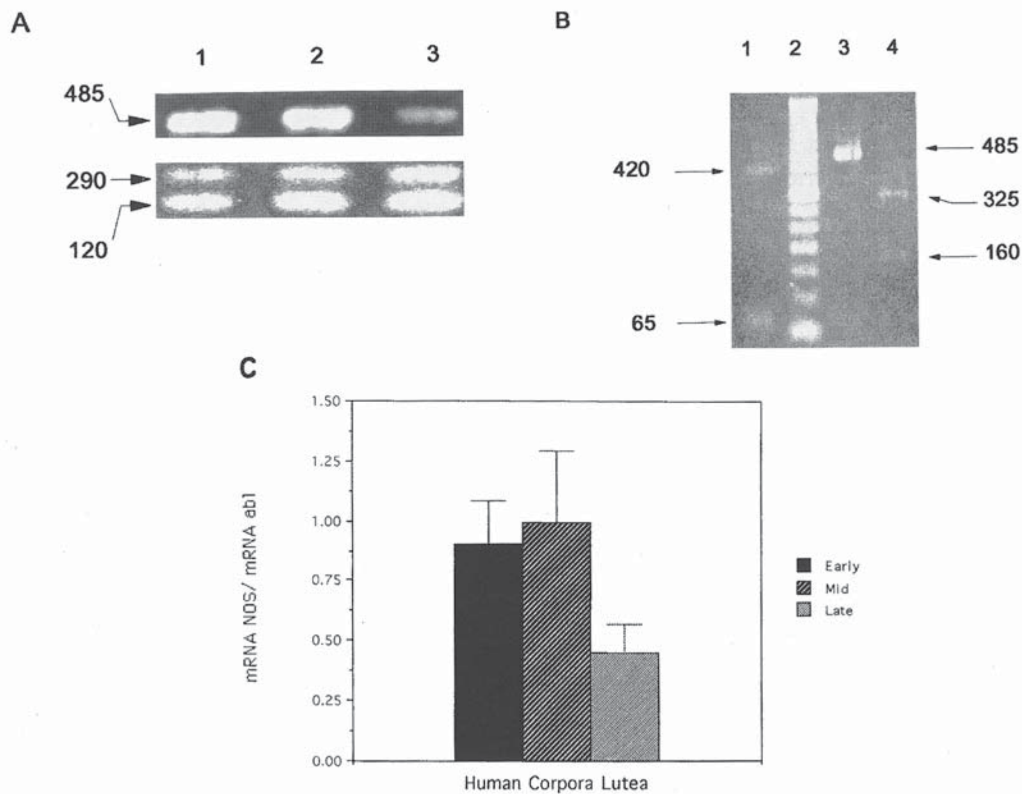

Fig. 1. (A) Expression of eNOS in human corpora lutea obtained at different stages of the luteal phase, by RT-PCR. PCR amplification of reverse-transcripted RNA from human CL of different ages using primers for eNOS (485-bp), abl (290-bp), or β_2-microglobulin (120-bp). Lane 1, early CL cDNA; lane 2, mid-CL cDNA; lane 3, late CL cDNA. Results are representative of 3 experiments with different human CLs. **(B)** Enzymatic digestion of PCR products. NOS amplified from mid-CL was digested with *Pst*I (recognizes position 1351 of the amplified product) or *Rsa*I (recognizes position 1091 of the amplified product). Lane 1, *Rsa*I digestions of the PCR product demonstrating the expected 420- and 65-bp product; lane 2, 50-bp DNA ladder; lane 3, the expected 485-bp product; lane 4, *Pst*I digestion of the PCR product demonstrating the expected 325- and 160-bp product. **(C)** Normalized yield NOS PCR products relative to abl PCR products. PCR amplification of NOS and abl-specific sequences were performed in the same condition. Results are the mean ± SEM of 4 experiments with 2 different human CLs.

respectively (Fig. 1A). The polymerase chain reaction (PCR) amplification products of abl and β_2-microglobulin are used as internal controls, since the genes for both proteins are expressed in all cells. Confirmatory evidence for the 485-bp amplification product was obtained by *Pst*I or *Rsa*I digestion of the PCR product, which generated two expected bands (*Pst*I, 325- and 160-bp; *Rsa*I, 420- and 65-bp), indicative of the endothelial constitutive NOS (Fig. 1B). No homology was observed with inducible or macNOS cDNA obtained from GeneBank and analyzed by the MacVector 4.1.1 Program. Nevertheless, the amplified product was not validated by direct sequencing. As observed in Fig. 1C, NOS expression, normalized to abl expression, was importantly diminished (64%) in late relative to mid-human CL. Meanwhile in the early stage, NOS was expressed to a lesser extent (12%) than in the mid-CL.

Figure 2A,B shows histological sections of human CL stained with hematoxylin/eosin. The parenchyma of the gland is composed of polygonal luteal cells with abundant, pale eosinophilic cytoplasm that may contain numerous lipid droplets. The spherical nucleus contains one or two large nucleoli. The immunohistochemical study (Fig. 2D–I) showed that both eNOS and macNOS were expressed in the human CL, with a greater number of cells expressing eNOS relative to those expressing macNOS. The positive staining for eNOS isoform was largely distributed within the parenchyma of the CL (Fig. 2D,E), indicated by the abundant punctuate positive staining in their cytoplasm. Cells

Fig. 2. *(opposite page)* Immunohistochemical localization of the distinct NOS isoforms in human CL: **(A,B)** Paraffin sections (4 µm) of human CL stained with hematoxylin/eosin (magnification, ×100 and ×400, respectively) showing steroidogenic cells (single small arrow) and a capillary (single arrow). **(C)** Negative control with normal mouse serum used in place of the respective primary antibody. **(D,E,F)** Luteal cells demonstrating abundant punctuate staining in their cytoplasm (D,E, double small arrow) and in endothelial cells from blood vessels (F, arrowhead) when using an MAb against eNOS (ECNOS). Cell nuclei are stained with hematoxylin (magnification, ×100, ×400, ×400, respectively). **(G,H,I)** Luteal cells showing punctuate staining in their cytoplasm, indicating the presence of macNOS when using the specific MAb (single arrow) (magnification, ×400, ×1000, ×1000, respectively). Scale bar: **(A–D)** = 100 µm; **(B,E–I)** = 10 µm.

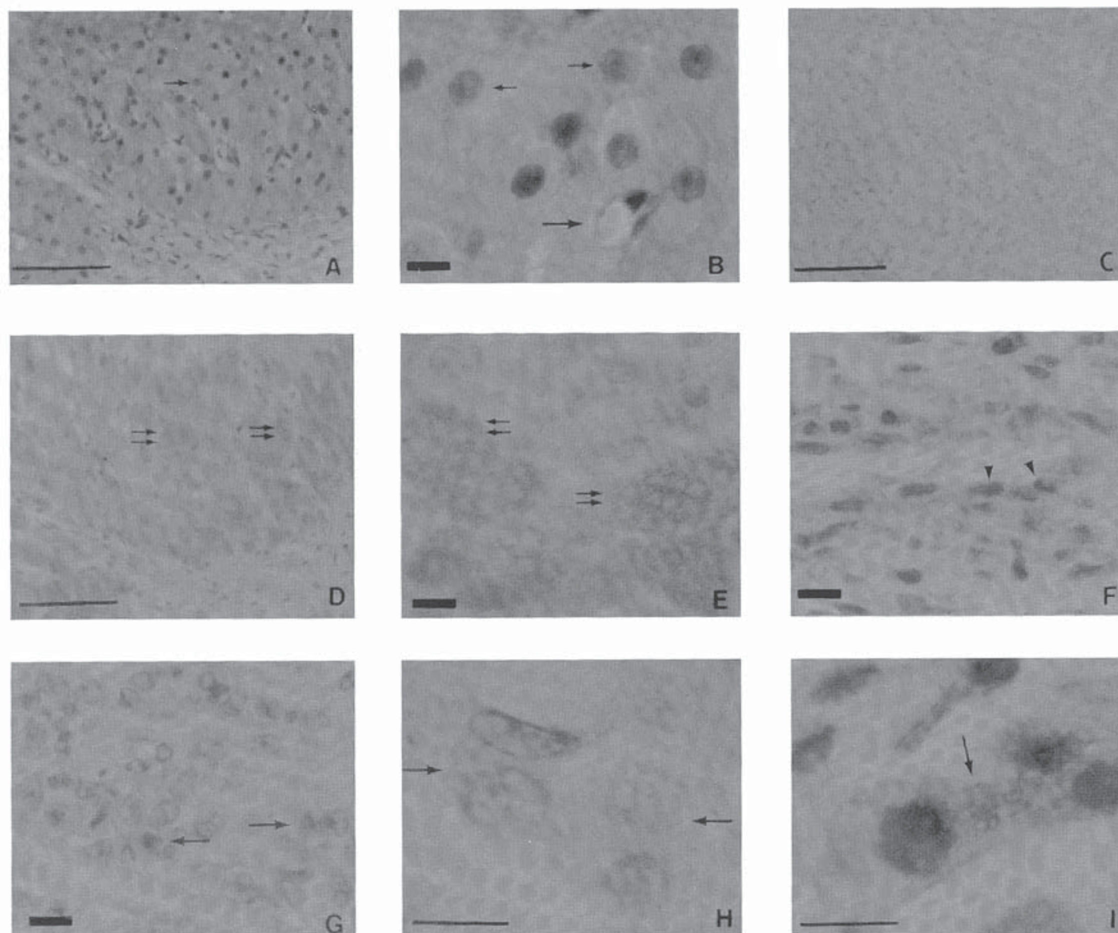

Fig. 2.

expressing eNOS isoform present a large cytoplasm and a prominent nucleus. Likewise, positive staining for this isoform was also observed within the endothelium of luteal blood vessels (Fig. 2F). On the other hand, punctuate staining for macNOS was also seen within the CL, although limited to only a few luteal cells. The cells expressing macNOS contained narrow cytoplasm (Fig. 2I) and appeared to be located near the capillaries.

We also studied the steroidogenic response to the action of NO· by treating mid-human luteal cells with increasing concentrations of the NOS substrate, L-Arg (Table 1). A dose-dependent decrease in estradiol production was observed at concentrations as low as 1.0 μmol/L, presenting L-Arg a significant inhibitory effect ($p < 0.05$) on both steroids at 1.0 mmol/L. In fact, the addition of L-Arg (1.0 mmol/L) to the cultures (Fig. 3) caused a significant decrease ($p < 0.05$) in progesterone (25%), testosterone (36%), and most importantly, in estradiol production (51%), as compared to the basal values. Similar results were obtained with sodium nitroprusside (SNP), an NO·-generating compound, as shown in Fig. 3.

Table 1

Dose-Dependent Effects of L-Arg, NO·-Generating Compound on Basal Levels of Progesterone and Estradiol in Dispersed Mid-Human Luteal Cells[a]

	Progesterone, ng/10^6 cells	Estradiol, pg/10^6 cells
Basal	85.9 ± 8.0	335.3 ± 41.8
L-Arg		
10 nmol/L	84.1 ± 9.3	328.7 ± 37.5
1.0 μmol/L	76.5 ± 8.3	231.0 ± 25.8[b]
100 μmol/L	68.8 ± 8.4	178.2 ± 22.1[b]
1.0 mmol/L	64.4 ± 8.2[b]	163.1 ± 24.5[b]

[a]Cells obtained from mid- ($n = 3$) human CL were treated with different concentrations of L-Arg for 24 h. Progesterone and estradiol production are expressed as mean ± SEM, and each experiment was performed in duplicate. Increasing concentrations of L-Arg diminished estradiol and progesterone production in a dose-dependent manner.

[b]$p < 0.05$ vs basal values.

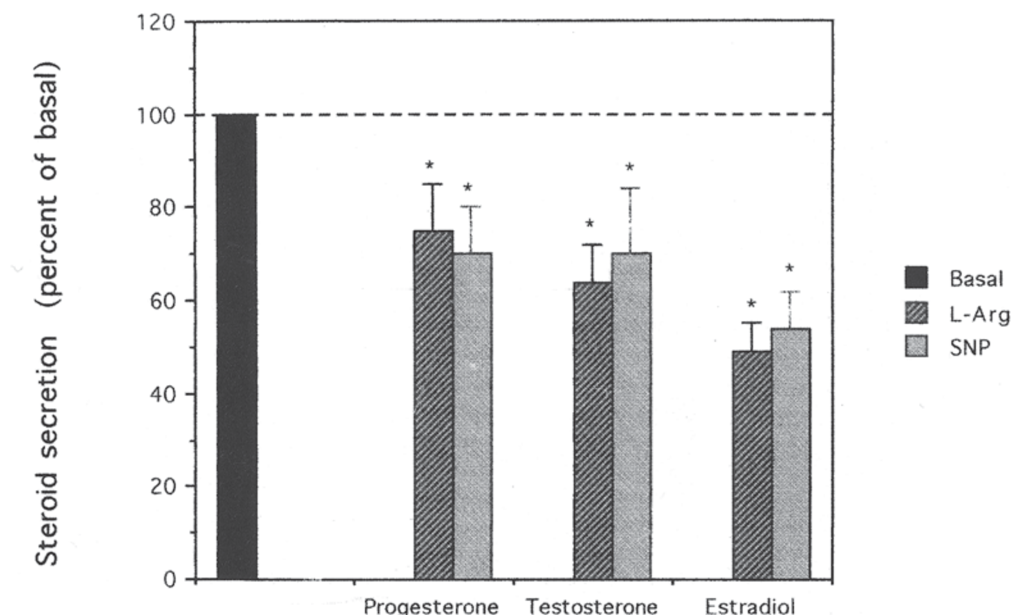

Fig. 3. Effect of NO·-donating compounds, L-Arg (NOS substrate) and SNP (NO·-generating drug), on progesterone, testosterone, and estradiol basal secretion by human mid-luteal cell cultures. Luteal cells were precultured in medium M-199, as described in Materials and Methods, and after 24 h, the cells were cultured in the presence of 1.0 mmol/L L-Arg or 1.0 mmol/L SNP for 24 h in L-Arg-free medium. Progesterone, testosterone, and estradiol levels are expressed as a percentage of basal values (basal = 0 dose of NO·-donor compounds; mean ± SEM). Each treatment was conducted in duplicate, and the experiment was repeated seven times. Basal values for progesterone, testosterone, and estradiol were 85.9 ± 8.0 ng/10^6 cells, 2.3 ± 0.4 ng/10^6 cells, and 335.3 ± 41.8 pg/10^6 cells, respectively. *$p < 0.05$ vs basal values.

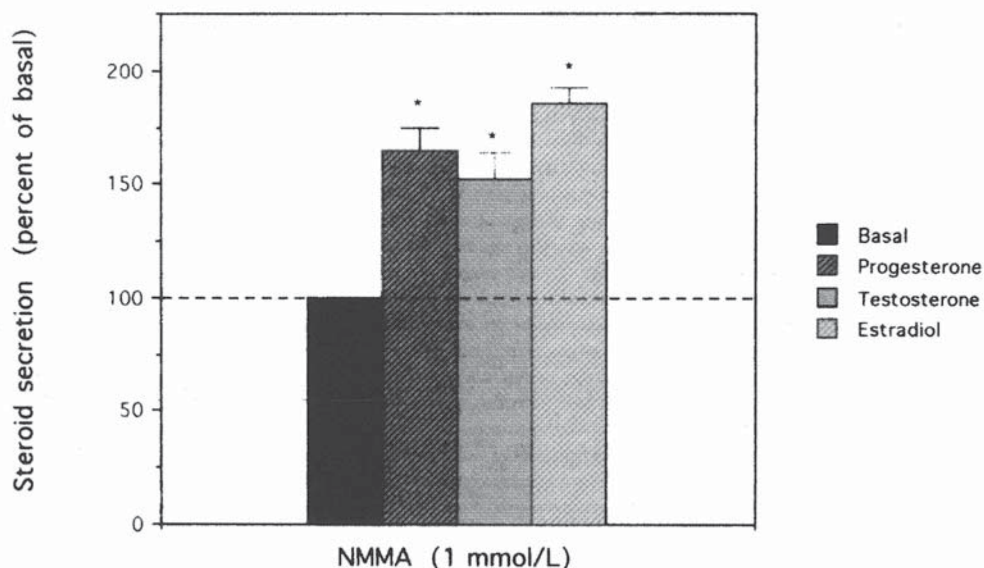

Fig. 4. Effect of endogenous NO· on human luteal steroidogenesis by cultured mid-luteal cells. Adherent human mid-luteal cells were treated with 1.0 mmol/L L-NMMA, specific NOS inhibitor, for 24 h as described in Materials and Methods. Results are expressed as percentages of basal values for progesterone (85.9 ± 8.0 ng/10^6 cells), testosterone (2.3 ± 4.0 ng/10^6 cells), and estradiol (335.3 ± 41.8 pg/10^6 cells). *$p < 0.05$ vs basal values.

To examine the effect of endogenous NO· on luteal steroidogenesis N^w-monomethyl-L-arginine (L-NMMA) (1.0 mmol/L), a specific NOS inhibitor, was added to some cultures (Fig. 4). The results show that progesterone, estradiol, and testosterone basal production were significantly enhanced ($p < 0.05$) by 65-, 80-, and 85%, respectively, in human mid-luteal cell cultures. Similar results were obtained with another NOS inhibitor, N^w-nitro-L-arginine methyl esther (L-NAME) (data not shown).

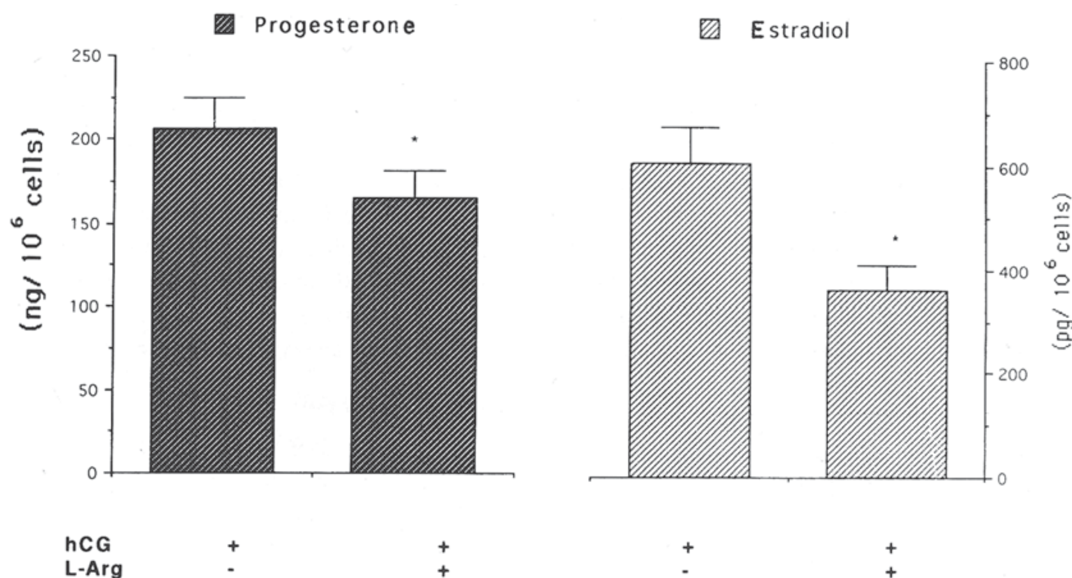

Fig. 5. Influence of NO· on the steroidogenesis of hCG-treated human mid-luteal cells. Luteal cells were treated with hCG (10 IU/mL) alone or in combination with L-Arg (1.0 mmol/L). Values are mean ± SEM of seven experiments, each performed in duplicate. Stimulated estradiol production was significantly diminished by the effect of L-Arg. *$p < 0.05$ vs hCG-treated condition.

We also examined the influence of NO· on steroidogenesis of human chorionic gonadotropin (hCG)-treated luteal cells, by culturing the mid-luteal cells either with hCG alone or in combination with L-Arg (1.0 mmol/L) (Fig. 5). Production of estradiol on the hCG plus L-Arg-treated cell cultures was significantly lower (40%, $p < 0.05$) than in cells cultured with hCG alone; meanwhile, progesterone synthesis showed a moderate decrease (20%) under these conditions.

Discussion

It is well accepted that NO· exerts an important regulatory role on a variety of physiological processes, including endothelium-dependent vascular relaxation, neurotransmission, and immunocytotoxicity *(8,9,17)*. In addition, it has recently been suggested that NO· regulates several aspects of the reproductive process *(18)*. The purpose of the present study was to determine whether NO· participates as a local regulatory messenger molecule on human luteal steroidogenesis. The fact that the human CL is heterogenous in its cell composition with an important percentage of nonsteroidogenic cells, including leukocytes *(3)* and endothelial cells, raises the possibility of intraluteal NO· production. To answer this question, we first investigated the expression of NOS in human CL obtained at different stages of the luteal phase. Our results showed that eNOS mRNA is expressed in the human CL during its life-span, in an age-dependent manner, being highest in CL obtained during the mid-luteal phase, in agreement with the vascular development of the gland, which is maximum during the mid-luteal phase. Also, both NOS isoforms are detected in the human CL parenchyma with a greater number of cells expressing eNOS, but macNOS is restricted to a few cells. It is most likely that eNOS isoform is expressed in steroidogenic cells, as well as in endothelial cells, whereas macNOS expression is apparently in nonsteroidogenic luteal cells, including macrophages, indicated by the morphological characteristics and the localization of the positive-stained cells. To our knowledge, this is the first study to report the expression of eNOS mRNA in human CL of different ages and the presence of both NOS isoenzymes in the human CL; however, these data are consistent with previous studies showing that eNOS is present in human granulosa cells *(16)* and also in rat luteinized ovaries *(18)*.

Based on in vitro observations in rat ovaries *(18)*, it has been suggested that NO· participates in functional luteal regression by inhibiting steroidogenesis, preferentially on estradiol synthesis. Similar results were found in granulosa/lutein cells *(16)*, where NO· directly inhibits the cytochrome P_{450} aromatase enzyme activity. The data obtained in the present study are in agreement with these reports, estradiol being the steroid most affected in the human CL by the action of NO·, independently from the source of NO·, SNP, or L-Arg. Moreover, our experiments also demonstrate an inhibitory effect of NO· on testosterone production, suggesting that the $P_{450\ c17}$ enzyme complex may be affected in a similar manner as other steroidogenic enzymes by the action of NO·, as previously reported *(19)*. Collectively, these data suggest that in human luteal cells, NO· may represent a multifunctional regulatory molecule, being responsible for the adequate blood supply to the CL, and therefore, for the steroid secretion rate, in addition to its function as an in vitro antisteroidogenic agent. These findings are consistent with the observations that NO· may

negatively regulate steroidogenesis in the rodent testes *(20)*, cultured Leydig cells *(21)*, and human granulosa/lutein cells *(16)*, as already discussed. Furthermore, in our studies, the inhibition of endogenous NO· generation by specific NOS inhibitors, resulted in a significant increase of the steroids produced by luteal cells, supporting the proposed modulatory role of NO· on human in vitro luteal steroidogenesis. On the other hand, the antisteroidogenic effect of NO· was also observed in the hCG-stimulated condition, indicating that NO· may affect the steroidogenic pathway at several cellular levels, at hCG receptor, and at post-hCG receptor sites.

In summary, the present investigation provides convincing evidence that NO· exerts a regulatory effect on cultured luteal cell function. Several findings support this observation, including the expression of eNOS mRNA in human CL of different ages, the presence of both NOS isoforms, the inhibitory effect of NO· on the steroidogenic pathway of human mid-CL, especially on estradiol production, and the increased steroid synthesis in the presence of NOS inhibitors. These results further suggest that NO· produced endogenously may act, by an autocrine and/or paracrine mechanism, as an antisteroidogenic agent in these cells. Taken together, these data are in agreement with our previous studies *(2,3)*, suggesting the participation of ROS in human luteal regression and the importance of cell-to-cell interaction in the normal function of the gland. Nevertheless, more studies are in progress to elucidate the mechanism of action of this agent and whether NO· is acting in an autocrine or a paracrine manner.

Materials and Methods

All chemicals, culture media, and hormones used were obtained from Sigma Chemical Co. (St. Louis, MO), except for the lymphocyte separation medium (Litton Bionetics, Kensington, MD) and collagenase (Worthington Biochemical Corp., Freehold, NJ). Monoclonal antibodies (MAbs) were purchased from Transduction Laboratories (Lexington, KY), and the second Ab and reagents for the detection system, from Dako Corp. (Carpinteria, CA). Primers, DNase I (Amp Grade), SuperScript RT II, random primers, *Rsa*I, *Taq* DNA polymerase, and guanidinium thiocyanate, were purchased from Gibco BRL Life Technology (Bethesda, MD). *Pst*I was obtained from New England Biolabs (Beverly, MA). ^{32}P-dCTP (SA 3000 Ci/mmol) was purchased from DuPont NEN Research Products (Boston, MA).

Subjects

Corpora lutea were obtained from 12 normal women, aged 30–36 yr, requesting surgical sterilization at the San Borja-Arriaran Clinical Hospital. The procedure was carried out as reported earlier *(22)*. Briefly, the minilaparotomy performed in women with previous cesarean sections was scheduled at different stages of the luteal phase: <5 d after the luteinizing hormone (LH) peak (early, $n = 2$), 5–9 d after the LH peak (mid-CL, $n = 7$), and >9 d after the LH peak (late, $n = 3$). All subjects gave informed consent for removal of the CL, which was approved by the Institutional Ethical Committee. After removal, the tissue was placed in sterile 0.15 *M* NaCl and transported to the laboratory at room temperature. The phase of the menstrual cycle of each woman was confirmed by endometrial and CL-dating biopsies *(23,24)*, and by measurements of progesterone concentration in plasma as previously reported *(3)*.

Expression Studies

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction *(25)*. Briefly, frozen early ($n = 2$), mid- ($n = 3$), and late ($n = 3$) human CLs were homogenized in a guanidinium thiocyanate solution, and extracted twice with phenol-chloroform-isoamilic alcohol. The aqueous phase was precipitated with isopropanol alcohol at –20°C. Total RNA was resuspended in diethylpyrocarbonate–water, and the RNA integrity was assessed by electrophoresis of an aliquot of each sample in a 1% agarose gel. Complementary DNA (cDNA) was synthesized by using SuperScript II reverse transcriptase (RT) and random primer as described previously *(26)*. A 485-bp PCR fragment was generated from human CL cDNAs with specific eNOS primers and PCR condition reported previously by Van Voorhis et al. *(16)* and modified by us, using a nucleotide mixture containing 0.4 µCi ^{32}P dCTP in The Minicycler, model PTC-150-16 (MJ Research Inc., Watertown, MA). To verify that the PCR-generated band was eNOS cDNA, purified PCR product was digested with 20 IU *Pst*I or *Rsa*I and resolved in 2% agarose-gel electrophoresis. As internal controls, we used primers that amplified β_2-microglobulin and abl mRNAs, both of which appear to be expressed ubiquitously *(27,28)*. The presence of a 120- and 290-bp amplification products confirms that the reaction contained intact β_2-microglobulin and abl mRNAs, respectively. Products were resolved on a 1.5% agarose-gel electrophoresis using ethidium bromide staining. Each amplified fragment and the negative water control corresponding to the bp bands were excised, and the radioactivity determined in a scintillation counter. The cpm obtained were corrected with the negative water control cpm, as was described previously *(26)*. The counts obtained from eNOS products were normalized with the counts obtained from abl PCR products. Both eNOS and abl cDNA amplifications were within the linear range.

Immunohistochemical Detection of NOS Isoforms

The detection of NOS isoforms was carried out in histological paraffin sections (4 µm) of human mid-CL by the avidin–biotin immunohistochemistry method *(3)*. The MAb used against eNOS (anti-ECNOS, N30020, Clone 3) was raised in mice against a 20.4-kDa protein fragment

corresponding to amino acids 1030–1209 of human eNOS. The MAb used against inducible or macNOS isoform (anti-macNOS, N32020, Clone 6) was raised in mice against a 21-kDa protein fragment corresponding to amino acids 911–1144 of mouse macNOS. The second antibody used was a biotinylated antimouse immunoglobulin raised in rabbits. The reaction was evidenced by avidin–biotin conjugated peroxidase using diaminobencidine as the chromogen, and cell nuclei were stained with hematoxylin.

Cell Dispersion and Culture

For experiments assessing the influence of NO· on steroidogenesis, cells from human mid-CL were dispersed as described previously *(29)*. Briefly, the luteal tissue was enzymatically dissociated in Medium 199 containing $NaHCO_3$ (26 mmol/L), BSA (0.1% w/v), HEPES (25 mmol/L), antibiotics (100 IU/mL penicillin and 5 mg/L streptomycin), collagenase (370 IU/100 mg tissue), and DNase (14 KU/100 mg tissue). Cells were counted in a hemocytometer, and viability was >85% during the time culture and in the presence of the different agents, as assessed by the trypan blue exclusion method. The same percentage of steroidogenic cells (3β-HSD positive) was observed in all cultures. Luteal cells (10^5 cells/well) were cultured in the defined *M* 199 at 37°C in a 5% CO_2-air atmosphere, as reported earlier *(29)*. After 24 h, adherent cells were washed and M 199 was replaced by Hank's (L-Arg-free) supplemented with gluthamine (0.1 mg/ml), BSA, $NaHCO_3$, HEPES, and antibiotics. Then, hCG (10 IU/mL), NO·-donating drug sodium nitroprusside (SNP, 1.0 mmol/L), NOS substrate L-Arg (10 nmol/L to 1.0 mmol/L), or competitive inhibitors of NOS NAME (1.0 mmol/L) and NMMA (1.0 mmol/L) were added to some plates. Cultures were terminated at 24 h and the media were stored at –20°C until assayed for progesterone, estradiol, and testosterone by specific radioimmunoassay as reported previously *(30)*. Steroid production was normalized to 10^6 viable luteal cells. Details of each experimental design are indicated in the figure legends.

Statistical Analysis

These data are presented as means ± 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-square difference multiple-comparison test *(31)*. A p value of < 0.05 was considered statistically significant.

Acknowledgments

This research was supported by FONDECYT 1950669. The authors wish to thank Iván Retamales and Fernando Gabler for performing the histological dating of the endometrium and corpora lutea and immunohistochemical evaluation, Natacha Salgado for preparing the manuscript, and the National Hormone and Pituitary Program (NIADDK), Baltimore, MD, for providing the reagents for LH assay.

References

1. Carlson, J. C., Wu, X. M., and Sawada, M. (1993). *Free Radical Biol. Med.* **14,** 78–84.
2. Vega, M., Carrasco, I., Castillo, T., Troncoso, J. L., Videla, L. A., and Devoto, L. (1995). *J. Endocrinol.* **147,** 177–182.
3. Vega, M., Castillo, T., Retamales, I., Las Heras, J., Devoto, L., and Videla L. A. (1994). *Free Radical Biol. Med.* **17,** 493–499.
4. Peperell, J. R., Wolcott, K., and Behrman H. R. (1992). *Endocrinology* **130,** 1001–1008.
5. Kirsch, T. M., Friedman, A. C., Vogel, R. C., and Flickinger, G. L. (1981). *Biol. Reprod.* **25,** 629–638.
6. Grossman, A. (1994). *Endocrinology* **134,** 1003–1005.
7. Ignarro, L. J. (1990). *Annu. Rev. Pharmacol. Toxicol.* **30,** 35–60.
8. Moncada, S., Palmer, R. M., and Higgs, E. A. (1991). *Pharmacol. Rev.* **43,** 109–142.
9. Nathan, C. (1992). *FASEB J.* **6,** 3051–3064.
10. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder S. H. (1991). *Nature* **351,** 714–718.
11. Marletta, M. A. (1994). *Cell* **78,** 927–930.
12. Knowles, R. G. and Moncada, S. (1994). *Biochem. J.* **298,** 249–258.
13. Moretto, M., Lopez, F. J., and Negro-Vilar, A. (1993). *Endocrinology* **133,** 2399–2402.
14. Yallampalli, C., Byam-Smith, M., Nelson, S. O., and Garfield, R. E. (1994). *Endocrinology* **134,** 1971–1974.
15. Salvemini, D., Misko, T. P., Masferrer, J. L., Siebert, K., Currie, M. G., and Needleman, P. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 7240–7244.
16. Van Voorhis, B. J., Dunn, M. S., Snyder, G. D., and Wiener, C. P. (1994). *Endocrinology* **135,** 1799–1806.
17. Moncada, S. (1992). *Acta Physiol. Scand.* **145,** 201–227.
18. Olson, L. M., Jones-Burton, C. M., and Jablonka-Shariff, A. (1996). *Endocrinology* **137,** 3531–3539.
19. Wink, D. A., Osawa, Y., Darbyshire, J. R., Jones, C. T., Eshenaur, S. C., and Nims, R. W. (1993). *Arch. Biochem. Biophys.* **300,** 115–123.
20. Adams, M. L., Mock, B., Truong, R., and Cicero, T. J. (1992). *Life Sci.* **50,** 35–40.
21. Welch, C., Watson, M. E., Poth, M., Hong, T., and Francis, G. L. (1995). *Metabolism* **44,** 234–238.
22. Vega, M., Devoto, L., Navarro, V., Castro, O., and Kohen, P. (1987). *J. Clin. Endocrinol. Metab.* **65,** 747–752.
23. Noyes, R. W., Hertig, A. T., and Rock, J. (1950). *Fertil. Steril.* 1, 3–12.
24. Corner, G. W. (1956). *Am. J. Anat.* **98,** 377–401.
25. Chomczynski, P. and Sacchi, N. (1987). *Biochem. J.* **162,** 156–159.
26. Johnson, M. C., Vega, M., Vantman, D., Troncoso, J. L., and Devoto, L. (1997). *Mol. Hum. Reprod.* **3,** 663–668.
27. Noonan, K. L., Beck, C., Holzmayer, T. A., Chin, J. E., Wunder, J. S., Andrulis, I. L., et al. (1990). *Proc. Natl. Acad. Sci. USA* **87,** 7160–7164.
28. Kohler, S., Galili, J. L., Donion, T. A., Blume, K. G., and Cleary, M. L. (1990). *Leukemia* **4,** 541–547.
29. Vega, M., Devoto, L., Castro, O., and Kohen, P. (1994). *J. Clin. Endocrinol. Metab.* **79,** 466–469.
30. Carrasco, I., Troncoso, J. L., Devoto, L, and Vega, M. (1996). *Hum. Reprod.* **11,** 1609–1614.
31. Milton, J. S. and Tsokos, J. O. (1987). *Statistics for Biology and Health Sciences*. Interamerican McGraw-Hill Editorial, Madrid.