# NITRIC OXIDE STIMULATES THE PRODUCTION OF ATRIAL NATRIURETIC PEPTIDE AND PROGESTERON BY HUMAN GRANULOSA LUTEINIZED CELLS WITH AN ANTIAPOPTOTIC EFFECT

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**Objective.** To study the effect of nitric oxide (NO) on atrial natriuretic peptide (ANP) and progesterone (P) production by human granulose luteinized cells (GLC) *in vitro* and to elucidate their role on the survival of cultured cells.

**Methods.** Human GLCs were cultured in HAM's F10/10 % FCS as monolayers for 24 h. Subsequently GLCs were treated for 24 h with 0.5 mM *Sodium Nitroprusside* (SNP, NO donor) and 0.5 mM Aminoglutethimide (AG , P450scc inhibitor). The levels of ANP and P were measured in supernatants of cultured cells by proANP(1-98) kit and RIA, respectively. Caspase-3 activity was determined by Ac-DEVD-pNA as substrate.

**Results.** The production of ANP and P was increased by NO as compared to control cells ($p<0.05$). AG diminished the production of P compared to SNP ($p<0.05$). The caspase-3 activity was significantly lower in SNP treated cells ($p<0.05$) and increased significantly after AG treatement compared to control cells ($p<0.05$).

**Conclusion.** NO generated by SNP in human GLCs culture stimulated the production of ANP and P. The higher levels of ANP and P were closely related to significantly lower caspase-3 activity thus showing the role of ANP, P and NO on the survival of preovulatory human follicle.

**Key words:** ANP – Apoptosis – Granulosa luteinized cells – Nitric oxide – Progesterone

Granulosa cells (GCs) play a crucial role in follicular development, corpus luteum formation, and function. Several endocrine, paracrine and autocrine factors play an important role in the regulation of GCs proliferation, differentiation and death. (**Richards and Hedin 1988;** Tilly et al. 1997). Apoptosis is one of the main types of cell death and involves an orchestrated series of biochemical events leading to characteristic changes in cell morphology and death. Atresia, which is accomplished by apoptosis at the cellular level, appears the most common fate of the developing follicles (Gougeon 1986). One of the major characteristics of GCs after LH receptor stimulation-luteinization is the ability to produce large amounts of P which is critical for the regulation of specific events such as ovulation and luteogenesis (Rondel 1974). Several studies demonstrated that progesterone shows a pleiotropic effects and plays the role of a survival factor in rat, porcine and human GCs (Svensson et al. 2000, 2001; Rueda 2000; Dineva et al. 2004, 2007; Makrigiannakis et al. 2000).

Natriuretic peptides (NPs) belong to a family of structurally related peptides and their properties include the

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modulation of water and salt homeostasis (Bold 1985), vasorelaxation (Needleman and Greenwald 1986) as well as the regulation of cell proliferation and differentiation (Hagiwara et al 1996; Komatsu et al. 1996). Actually, there exist three natriuretic peptides, such as atrial (ANP), brain (BNP), and C-type (CNP). The expression of NP components system, i.e. NPs and NP receptors (NPRs) has been demonstrated in the mammalian ovary (Gutkowska et al. 1993; Russinova et al. 2001). The changes of ANP expression during an estrous cycle have been detected in the rat ovary (Russinova et al. 2001; Kim et al. 1992) and ANP presence was demonstrated in the follicular fluid (Kim et al. 1989; Sundsfjord et al. 1989); granulosa cells (Pandey et al. 1987; Ivanova et al. 2003) and luteal cells (Vollmar et al. 1988).

From the physiological aspect, many data suggest that ANP influences the oocyte meiosis, inhibits the cumulus expansion as well as spontaneous oocyte maturation, plays an important role in the ovulation, ovarian steroidogenesis and can modulate follicular atresia/apoptosis (Dineva et al. 2007; Vollmar et al. 1988; Samson et al. 1988; Gutkowska et al. 1999; Tornell et al. 1990; Zhang et al. 2005). However, contradictory data exist on the positive relation between ANP and P production in the ovary (Dineva et al. 2004; Pandey et al. 1987; Steegers et al. 1990; Johnson et al. 1994). Diverse effects of NPs are mediated by the activation of membrane bound receptors with a particulate guanylate cyclase (GC) activity to generate cyclic guanosine monophosphate (cGMP) (Feil et al. 2003). Another pathway for cGMP generation is mediated by soluble guanylate cyclase (sGC) activated by NO. It is known that NO can influence different aspects of ovarian physiology such as ovulation, follicular development, oocyte maturation, steroidogenesis, apoptosis and several of such effects are mediated by the activation of soluble guanylate cyclases (sGC) followed by cGMP production (Tamanini et al. 2003). The activation of either form of guanylate cyclase results in a large increase in cGMP production and subsequent activation of cGMP dependent signaling pathways. Recently, LaPolt et al. (2003) reported that the components of cGMP signaling pathways are expressed in the ovary and that cGMP can modulate the GLCs function by a complex and diverse mechanisms.

The aim of this study was to investigate the effect of NO as donated by SNP on the production of atrial natriuretic peptide and progesterone by human granulosa luteinized cells in vitro, as well as to take up the survival role of ANP, P and NO on cultured cells.

## Materials and Methods

**Isolation of human GLCs.** GLCs were isolated from follicular fluids (FFs) obtained after ovum pick-up from at least three women undergoing an "In vitro Fertilization – Embryo Transfer" (IVF-ET) program in each of the three experiments, after informed consent was given. Controlled ovarian hormonal stimulation (COHS) was performed using a combination of GnRH agonist (Decapeptyl, Organon, Netherlands; 0.1 mg/d s.c.) in a short programming protocol and HMG (Humegon, Organon, Netherlands) and/or rFSH (Puregon, Organon, Netherlands) in a variable regimen (Repro-BioMed. Center, Sofia). Ethical approval of follicular fluids investigations was obtained by the local clinical Ethics committee.

After gradient centrifugation for 10 min at 200 x g on Histopaque-1077 (Sigma, Saint Louis, Missouri, USA), GLCs were aspirated from interphase and washed with culture medium (HAM's F-10/10 % fetal calf cerum (FCS; Sigma, Saint Louis, Missouri, USA). The cell viability was determined by the Trypan blue exclusion method.

**Preparation of *in vitro* culture of human GLCs.** The GLCs were diluted in HAM's F-10/10 % FCS supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) at $70x10^4$cells/ml and cultured in 24-well plates (Linbro, Flow Labs., Virginia, USA) 200 μl/well (for ANP and P measurement), and in 96/well plates (Linbro, Flow Labs, Virginia, USA), (50 μl/well) for caspase-3 activity study. The plates were incubated at 37 °C/5 % $CO_2$ and after 24h culture period the attachment of cells was already achieved. The medium was removed and replaced as follows: one part of the cells was supplemented with 0.5 mM SNP (Sigma, Saint Louis, Missouri, USA) in HAM's F-10/1 % FCS as described by Chun et al. (1995), while the other part was supplemented with 0.5 mM AG (Sigma, Saint Louis, Missouri, USA) according to Wang et al. (2006) in HAM's F-10/1 % FCS. Control cells were supplemented with HAM's F-10/1 % FCS only and all cells were cultured for next 24 h. At the end of the culture period supernatants were aspirated and stored at -20 °C (for the measurement of P) and at -80 °C (for the measurement of ANP). The 96-well plates were air dried and stored at -20 °C for caspase-3 activity assay.

**Progesterone assay.** Direct radioimmunoassay of progesterone concentration in cell supernatants was performed according routine method described else-

**Table1**
**ANP (fmol/ml) from *in vitro* cultured GLCs, treated with SNP, AG and control cells (mean ± SD, range (min-max) and significant difference between studied groups)**

| **GROUPS mean ± SD (range:min-max)** | **CONTROL** 0.105 ± 0.074 (0.101-0.109) | **+ AG** 0.175 ± 0.023 (0.169-0.186) | **+ SNP** 0.355 ± 0.0438 (0.345-0.367) |
|---|---|---|---|
| **+ SNP** 0.355 ± 0.0438 (0.345-0.367) | **p<0.05** | | |
| **CONTROL** 0.105 ± 0.074 (0.101-0.109) | | **p>0.05** | |
| **+ AG** 0.175 ± 0.023 (0.169-0.186) | | | **p<0.05** |

where (Todorov et al. 2002). 1,2,6,7-[$^3$H]-progesterone with specific activity of 85 Ci/mmol was purchased from Amersham (Pharmacia Biotech Ltd., UK). Sensitivity of the assay was 0.01 ng/ml, intra- and interassay coefficient of variation were 6.1 % and 10.2 %, respectively. For each experimental point three to four wells were set up for P determination and the primary obtained results were calculated as means ± SEM.

**Measurement of ANP concentration** in supernatants from *in vitro* cultured GLCs was performed using ProANP(1-98) kit (Biomedica, GRUPPE, Germany) according manufacturers instruction. Briefly, undiluted supernatants (20 µl/well) and a polyclonal proANP(85-90)-Biotin antibody (100 µl/well) were incubated simultaneously in 96 wells plates, precoated with an anti-proANP(10-19) antibody for 150 min at 37 °C/5 % $CO_2$. After washing with buffer further incubation for 60 min at 37 °C/5 % $CO_2$ with streptavidine-peroxidase conjugate (100 µl/well) was performed. The color reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) as substrate and the reaction was terminated with stop solution. The optical density (OD) was measured by Multiplate reader (LKB, Rome, Italy) at 450 nm and converted to fmol/ml ANP using a standard curve. The detection limit of ProANP(1-98) kit (Biomedica, GRUPPE,Germany) was 50 fmol/ml.

**Caspase-3 activity assay.** Cultured cells were lysed with a lysis buffer (50 mM Hepes, 100 mM NaCl, 0.1 % CHAPS, 1 mM EDTA, 10 mM DTT, 10 % Glycerol (Sigma, Saint Louis, Missouri, USA) . The soluble fraction of the cell lysate was assayed for caspase-3 activity using AcDEVD-Pna substrate (Sigma, Saint Louis, Missouri, USA). After incubation for two hours at 37 °C/5 % $CO_2$ the intensity of color reaction was measured by Multiplate reader (LKB, Rome, Italy) at 405 nm.

**Statistical evaluation.** Statistical evaluation was performed with software package STATISTICA 6.0 using one way ANOVA followed by Newman-Keul post hoc test. The results were expressed as mean± SD and significance was assumed at $p< 0.05$

## Results

**Effect of SNP and AG treatment on the production of ANP and P in GLCs culture.** The levels of ANP in supernatants (as fmol/ml) measured by proANP (1–98) kit as presented in Table 1 revealed that the production of ANP reached significantly highest level after SNP treatment as compared to control and AG treated cells ($p<0,05$). This showed a stimulatory effect of NO (as generated by SNP) on the production of ANP by human GLCs *in vitro*. However, the AG as an inhibitor of the synthesis of P450scc and P did not influence the production of ANP in cultured cells significantly (Table 1).

After treatment with SNP, the production of P by cultured GLCs was increased to the levels significantly higher than these in control cells ($p<0.05$; Table 2). When GLCs were cultured in the presence of AG, the production of P was strongly diminished compared to that obtained in SNP treated cells ($p<0.05$; Table 2) and was lower than that in control cells.

**Effect of SNP and AG treatment on the level of apoptosis of GLCs.** Using a specific caspase-3 substrate (AcDEVD-Pna) the activity of caspase-3 in lysates from GLCs at the end of a culture period was estimated. Significantly higher caspase-3 activity was found in the lysates of GLCs treated with AG (Table 3), compared to control cells ($p<0.05$) and also to SNP treated cells ($p<0.05$). This shows that the diminished P production (Table 2), as well as the lower ANP levels (Table.1) in cultured GLCs led to increased activa-

**Table 2**
**Concentration of P (ng/ml) from *in vitro* cultured GLCs, treated with SNP, AG and control cells (mean ±SD, range (min-max) and significant difference between studied groups)**

| GROUPS Mean ± SD (range: min-max) | CONTROL<br>0.785 ± 0.1202<br>(0.700-0.840) | + AG<br>0.575 ± 0.0353<br>(0.550-0.600) | + SNP<br>2.040 ± 0.098<br>(1.970-2.110) |
|---|---|---|---|
| **+ SNP** 2.040 ± 0.098 (1.970-2.110) | **p<0.05** | | |
| **CONTROL** 0.785 ± 0.1202 (0.700-0.840) | | **p>0.05** | |
| **+ AG** 0.575 ± 0.0353 (0.550-0.600) | | | **p<0.05** |

tion of caspase-3, the executor enzyme, and subsequent apoptotic death. Concerning SNP treated cells, in which the generation of NO was induced, and the increased concentration of ANP (Table1) and P (Table 2) were measured, significantly lower caspase-3 activity was demonstrated as compared to control and AG treated cells ($p<0.05$; Table3).

## Discussion

NO acts as a biologic mediator in the regulation of multiple physiological processes. NO has emerged as a novel regulator of ovarian functions, such as ovulation, steroidogenesis and apoptotic cell death (Hattori and Tabata 2006; Tamanini et al. 2003). The study by Jablonka-Shariff et al. (1999) showed that ovarian NO synthesis is required for maximal ovulation, and that a lack of NO during the periovulatory period results in some defects in oocyte maturation. It is feasible that the effects of NO are strongly dependent on the interactions with other growth modulating factors acting within the ovary and that locally produced NO contributes to modulate follicle development and possibly prevents the apoptosis (Tamanini et al. 2003).

In the ovary, the role of ANP in the follicle development, antral follicle growth, cumulus cell differentiation, steroid metabolism, luteinization, oogenesis and oocyte maturation has been demonstrated (Dineva et al. 2007; Vollmar et al. 1988; Samson et al. 1988; Gutkowska et al. 1999; Tornell et al. 1990; Zhang et al. 2005). The activation of GC-A receptor by ANP and that of sGC by NO results in cGMP production and its signaling pathways eliciting in the ovary (LaPolt et al. 2003). Enhancing effect of NO on the production of ANP could be explained by cGMP signaling. The results obtained in this study showed that ANP concentration in supernatants was significantly higher when GLCs were cultured in the presence of SNP, compared to the controls as well as to the cells treated with AG. In addition to the particulate GCs, the sGC represents a related cGMP producing enzyme, that is activated by NO (LaPolt et al. 2003). Sodium nitroprusside ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$) is the substance serving as a source of NO. Our results showed that NO donated by SNP is involved in the regulation of ANP production from human GLCs and we assume that this was probably realized by cGMP signaling pathway. Our assumption is in accordance with the results by Lenz et al. (1999) on the increase

**Table 3**
**Caspase-3 activity in GLCs treated with SNP, AG and control cells (mean ±SD, range (min-max) and significance difference between studied groups**

| GROUPS Mean ± SD (range: min-max) | CONTROL<br>0.1287 ± 0.0045<br>0.121-0.135 | + AG<br>0.1358 ± 0.009<br>0.118-0.149 | + SNP<br>0.1048 ± 0.0091<br>0.090-0.118 |
|---|---|---|---|
| **+ SNP** 0.1048 ± 0.0091 0.090-0.118 | **p<0.05** | | |
| **CONTROL** 0.1287 ± 0.0045 0.121-0.135 | | **p<0.05** | |
| **+ AG** 0.1358 ± 0.009 0.118-0.149 | | | **p<0.05** |

of ANP secretion from human kidney cell line (HEK 293) in the presence of NO donor - SNP. The latter authors documented the comparable effects between SNP and 8-Br-cGMP in the induction of ANP secretion by HEK 293 and suggested that NO stimulated sGC represents a system modulating the secretion of ANP in the kidney. We suppose that similar mechanism could be operating in GLCs.

NO may act to inhibit estradiol production in human granulosa cells (Van Voorhis et al. 1994) and also that of estradiol and androstenedione in rats (Dunnam et al. 1999), while Bonello et al. (1996) suggested that NO may stimulates estrogen synthesis. The effect of NO on P synthesis was investigated by Van Voorhis et al. (1994) and Kagabu et al. (1999) who showed that the administration of NO donor inhibited P production by one order of magnitude less than that of estradiol. The latter authors also confirmed that the effect of NO donors on ovarian aromatase is not dependent on the protein kinase A and must be related to NO release.

The results obtained in this study showed that the generation of NO by SNP treatment resulted in significantly elevated P production from GLCs, in comparison to control cells and cells cultured in the presence of P450 scc inhibitor (AG). In general, several NO actions are mediated by iron-containing enzymes: guanylate cyclase and cyclooxygenase (Salvemini 1997). Because cytochrome P-450 steroidogenic enzymes contain an iron–hem centre, it is most likely that the activity of this enzyme can be modulated by NO (Guengerich1989). Up regulation of P production in human GLCs treated with SNP-donor of NO suggested that NO may participate directly in the mechanism regulating the synthesis of steroids. Moreover, the increase of ovarian P production may result from enhanced bioactivity of enzymes involved in the signaling pathway of the conversion of cholesterol to P (Gore-Langton and Armstrong 1988). The ability of GLCs after LH receptor stimulation to produce large amounts of P as that in the case of human GLCs used in this study, would be mediated by NO (Rondell 1974). On the other hand, ANP mediated cGMP accumulation and enhanced P secretion in human (Tamanini et al. 2003) and rat (Johnson et al. 1994) granulosa luteinized cells were demonstrated. Thus, our results showed that NO results in increased ANP and P production in human GLCs thus suggesting the pleiotropic effect of NO in the ovary.

Progesterone is an autocrine/paracrine regulator of human GCs survival (Makrigiannakis et al. 2000) and plays the role of an important antiapoptotic factor in human, porcine and bovine GLCs (Rueda et al. 2000; Dineva et al. 2004, 2007). In the present study, the lower caspase-3 activity of cultured GLCs revealed that in the cases of up-regulated P and ANP production after SNP - NO donor treatment, the apoptosis of cells was inhibited significantly compared to down-regulated P production (after AG –P450scc inhibitor treatment). These data suggest that NO may stimulate the ANP and P production in human GLCs acting in autocrine manner and thereby preserving them from apoptosis. The anti-apoptotic cGMP pathway is regulated by multiple mechanisms at different stages of follicular development, including possible participation of as yet unidentified factors (Chun et al.1998). Thus, ANP as a physiological stimulator of guanylate cyclase, may prevent the apoptosis in addition to multiple other functions in ovarian follicles (Tornell et al. 1990) Our results showed that stimulated ANP and P production by NO inhibits caspase-3 activity in human GLCs.

In conclusion, NO generated by SNP in human GLCs culture stimulated the production of ANP and P. The increased levels of ANP and P were closely related to significantly lower caspase-3 activity showing the survival role of ANP, P and NO in preovulatory human follicle.

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