Nitric Oxide-Induced Apoptosis in Human Granulosa Cells

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Abstract

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Introduction: Recent evidence suggests that nitric oxide (NO) acts as an important factor in a variety of physiological and pathological processes, including reproductive function. The purpose of the present study was to investigate whether NO might significantly induce any apoptotic changes in cultured human granulosa cells.

Material and Methods: The granulosa cells (GC) were obtained from women taking part in an *in vitro* fertilization (IVF) program. After 48h culture, 1mM DETA/NO was added to the culture medium and then the apoptosis of granulosa cells was evaluated by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) immediately and after one hour culture.

Results: Nitric oxide significantly increased apoptotic index after one hour in human granulosa cell culture ($p<0.024$), but it did not significantly affect the controls and the group which apoptotic index was calculated immediately after NO donor addition.

Conclusions: These results suggest that, apoptosis of human granulosa cells is mediated by DETA/NO, and this effect is directly proportional to the duration of the exposure.

Keywords: Nitric oxide, apoptosis, granulosa cells

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Introduction

Apoptosis has been found to occur in three different ovarian cell types: oocytes, granulosa cells, and luteal cells. During folliculogenesis, apoptosis has been mainly observed in the granulosa cells of the growing follicles (1). It has been shown that the incidence of apoptotic granulosa cells is a very sensitive indicator to estimate the quality of follicles and oocytes in an IVF program (2). This process seems to be principally controlled by hormones. Testosterone induces and estradiol inhibits granulosa cell apoptosis in rodent ovaries (3).

On the other hand, it is established that Nitric Oxide, a free radical, (NO) has important physiological roles in a variety of reproductive processes such as follicular development and ovulation (4). Nitric oxide (NO) has recently emerged as a potential regulator of follicular development because of its involvement in the regulation of several physiological functions of the ovary (5).

Nitric Oxide has beneficial effects when generated within physiological limits. But when generated in large quantities for longer time, as during infection and immunological reactions, NO can cause cytotoxic effects (6). Nitric oxide is produced by conversion of L-arginine to L-citrulline by a family of enzymes known as NO synthase (NOS), which includes inducible NOS (iNOS), constitutive NOS (cNOS), and neuronal NOS (nNOS) (7). Inducible NOS (iNOS) is induced in a variety of cells,

including macrophages and other cell types, by exposure to cytokines, inflammatory factors, and hormones (8). These reactions produced large amounts of NO, which contribute to the host defense, as well as killing the cells in the region (9-14).

It has been shown that endogenously produced NO, as well as NO-releasing agents inhibit steroidogenesis in granulosa-luteal cells and the possible roles of the NO/NOS system in the control of ovarian functions are based on the modulating effects of NO/NOS-related agents of granulosa cells (GC) function (15).

In the ovary, NO can be produced by many different cell types, including vascular endothelial cells, macrophages, and mast cells in response to gonadotropins and other hormones (16). These cells are present in the ovarian interstitium, representing a potential source of NO release in this organ. Clinical studies have shown that pathological conditions including endometriosis and reproductive tract infection (male and female) are associated with infertility (14, 17). In both conditions, there are increases in the number of activated macrophages both in the pelvis and in the reproductive tract (17, 18), and these activated cells could produce large quantities of NO in addition to other effector molecules (14).

Large amounts of NO can promote breakage of DNA strands, which can initiate apoptosis and cause cell death in various cell types such as granulosa cells (12, 13, and 14). The mechanism of NO-induced apoptosis in cells involves an increase in the ratio of Bax/Bcl-2 gene expression, which leads to the release of cytochrome c from the mitochondria to the cytosol, finally activating caspase-3 and resultant DNA breakage and apoptosis (19).

The present study was performed to obtain more information about the effect of NO on apoptosis in human granulosa cells cultured *in vitro* with specific NO donor, DETA/NO.

Material and Methods

Isolation and culture of granulosa cells

Human granulosa cells were isolated from a total of 123 follicles obtained from 32 women undergoing *in vitro* fertilization (IVF) program in McMaster university's hospital as described previously (2, 3). Before follicular aspiration, the patients were treated with a gonadotropin-releasing hormone analogue to suppress endogenous gonadotropin secretion. Follicle-stimulating hormone (FSH) was used to induce the development of several follicles. Follicular aspirations were performed after 36-38 hours of administration of a single dose human chorionic gonadotropin (hCG).

After follicular aspiration, the cumulus-oocyte complexes were separated for IVF and the granulosa cells obtained from all follicles of several donors in each day were pooled and transported immediately to the laboratory. The granulosa cells were purified and cultured as described previously (20).

In brief, the cells were dispersed and separated from red blood cells. Then they were washed and plated with a medium containing Dulbecco's modified Eagle medium (DMEM) Hams F12 (1:1) supplemented with 10% fetal calf serum (Gibco, UK), 2mM L-glutamine, 100 IU/ml Penicillin, and 100 μ g/ml Streptomycin sulfate (Gibco) at a density of 2-5 $\times 10^5$ cell/well on 8-well plates. Each pooled group of granulosa cells was divided into 4 plates (groups) and cultured at 37°C in a 95% air/5% CO_2 CO_2 humidified environment for up to 48 hours.

After 48 hours of incubation:

- In group 1 (control-0), the cells were fixed with 10% formalin and placed on a slide. The slides stored in 100% methanol at 4°C until TUNEL assay.
- In group 2 (NO-0), 1 mM diethylenetriamine nitric oxide (DETA/NO), a long half-life of NO releaser (18) (Research Biochemical's International, Natick, MA), was added to the cells and they were immediately fixed and stored.
- In group 3 (control-1), the cells were cultured for one hour more without any (DETA/NO), and they were then fixed and stored.
- In group 4 (NO-0), the cells were incubated in a medium containing 1 mM (DETA/NO) for one hour, and they were then fixed and stored.

In situ 3′ end labeling of apoptotic cells

For detection of apoptotic cells, the TUNEL (Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling) technique was used according to the procedure advised in the Klenow-FragEL DNA fragmentation detection kit (Oncogen research, USA). In brief, the cells were

rehydrated by a short rehydration step and washed in PBS, to make them permeable. They were incubated with proteinase K (dilute 2 mg/ml proteinase K 1:100 in 10 mM Tris, pH 8) for 5 minutes at room temperature. Endogenous peroxidase was inactivated by H_2O_2 in methanol for 5 minutes at room temperature.

In situ end-labeling was performed using non-radioactive digoxigenin-dd UTP, a Klenow labeling reaction (Oncogen, USA). The slides were incubated with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-dd UTP at 37 °C for 1.5 hours in a humidified chamber, and $3'$-OH ends of DNA fragments were labeled with digoxigenin.

Then the slides were incubated with Stop Buffer and Blocking Buffer at room temperature for 5 and 10 minutes, respectively. After incubation with anti-digoxigenin antibody conjugated with peroxidase for 30 minutes at room temperature, the slides were washed 4 times within $1\times TBS$, and they were then stained using 0.05% diaminobenzidine (DAB) tetrahydrochloride for 10 minutes at room temperature. In the next step, the specimens were washed 4 times in distilled water (dH_2O) and counterstained with methyl green for 5 minutes. After dehydration and mounting, cover glasses were placed and the slides were examined under a light microscope. Then, TUNEL-positive cells were stained brown and negative cells stained green (Fig. 1). For evaluation of apoptosis, an Olympus B×50 microscope with a ×20X objective (Tokyo. Japan) was used.

A total of 1000 cells were randomly selected and the number of apoptotic cells was counted in each slide. The incidence of apoptosis was calculated as the proportion of apoptotic cells in 1000 cells (19). A total of 17 slides were prepared for each group. The proportion of apoptotic index for each slide and the mean of apoptotic index for each group were calculated.

Statistical analysis

Differences among the means of apoptotic cell indices were compared by one-way analysis of the variance coupled with Dunett 3 Post Hoch test using SPSS version 10. A p value of less than 0.05 was considered statistically significant.

Results

The results showed that the proportion of apoptotic cells in group NO-1 was (24.17%). It was significantly higher than the proportion of apoptotic cells in other groups ($p<0.024$). There were no statistically significant difference between the control groups (control-0 = 6.76% and control-1 = 8.19%) and the NO-0 group (= 13.15%) ($p>0.05$) (Fig. 1 and 2).

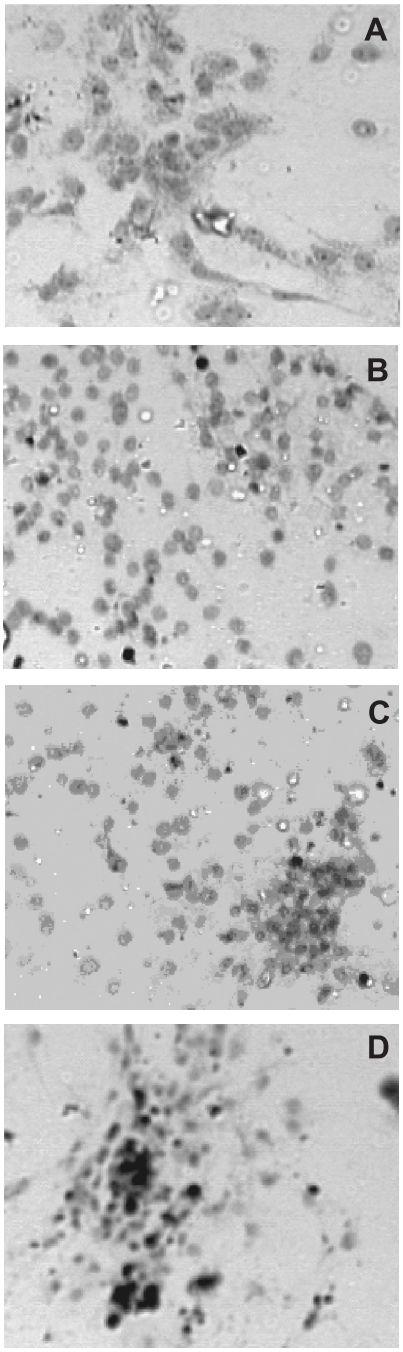

Figure 1. TUNEL staining of nuclei in human granulosa cell cultures (brown) and counterstaining with methyl green (green). A= control-0; B= NO-0; C= control-1; and D= NO-1.
(This figure has also been printed in full-color at the end of the issue.)

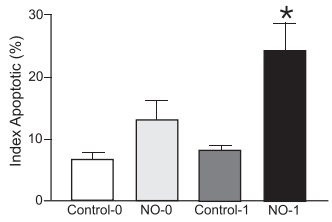

*Fig. 2. Effect of NO donor DETA/NO (1mM) on apoptosis of human granulosa cells immediately and after a 1 hour-culture. Results are shown as the mean ± standard error of the mean. * $P < 0.024$ versus control-0.*

Discussion

In the present study we provided some evidence that NO could induce toxicity and increase apoptosis in human granulosa cells. The results showed that treatment of granulosa cells with DETA significantly increased the proportion of apoptotic granulosa cells in a time-dependent manner, and the proportion of apoptotic cells after one hour increased to about three-fold the control.

NO is known to be an important mediator in several physiological systems (21) and has beneficial biological effects when generated within physiological limits. When generated in large quantities for long periods, mainly during immunological reactions, NO is cytostatic and cytotoxic for invading microorganisms and might also damage the tissues that generate it (22). Clinical studies have shown that reproductive tract infection both in females and males is associated with increased incidence of infertility (23). So, we assume that pathologically increased NO synthesis might, at least in part, be responsible for causing infertility in infected patients, possibly by increased apoptosis in granulosa-luteal cells. Although it has been shown in a study that NO directly inhibits spontaneous apoptosis in cultured granulosa cells (24), several studies have dealt with the relationship between NO and apoptosis in a diverse range of tissues and cells (25, 26), which is in concordance with our data, documenting both pro- and anti-apoptotic effects of NO. Higher concentrations of NO, which generally are observed in pathological settings, may induce apoptosis (27), but lower amounts prevent apoptosis.

Previous studies have indicated that the inhibitory effect of NO on FSH-induced aromatase activity is associated with inhibition of FSH-stimulated cAMP (cyclic adenosine monophosphate) rise in granulosa cell cultures. A dose of DETA/NO that maximally inhibited FSH-stimulated E2 production resulted in a 43% reduction in cAMP. This inhibitory effect of NO on cAMP accumulation is consistent with decreasing cAMP levels in cultured human granulosa-luteal cell (28). The mechanism through which NO inhibits cAMP levels in granulosa cells is not clear. Previous reports demonstrate direct inhibitory effects of NO on two isoforms of adenylyl cyclase (29). Thus, NO may antagonize FSH-induced cAMP production by direct interactions with adenylyl cyclase. On the other hand, it is known that cGMP (cyclic guanosine monophosphate) induces activity of a cGMP-stimulated phosphodiesterase (PDE2) that increases the hydrolysis of cAMP (30). For instance, the inhibitory effect of atrial natriuretic peptide on adrenal aldosterone production and cAMP levels is related to the induction of PDE2 (31). NO may also influence follicular development by mediating the effects of gonadotropins on the blood-follicle barrier, thus influencing its permeability to different substances (32). The overall results on the effects of NO on folliculogenesis suggest that locally produced NO contributes to modulate follicular development and possibly prevents apoptosis at least at low concentrations, whereas high levels may promote cell death via peroxynitrite formation.

Conclusion

This study provides convincing evidence that nitric oxide is involved in granulosa cell apoptosis and plays a crucial role in reproductive processes, even though most studies have been carried out in rat and human beings, and very little is known about live stock.

Fine-tuning of nitric oxide generation seems to be essential for ovarian physiology. However, the precise mechanisms by which it exerts its effect are not clearly understood and need further investigation. Future studies should also be aimed at verifying whether ovarian dysfunctions are associated with an altered nitric oxide production in order to clarify whether these defects can be corrected by nitric oxide.

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