Nitric Oxide Mediates Interleukin-1–induced Cellular Cytotoxicity in the Rat Ovary

A Potential Role for Nitric Oxide in the Ovulatory Process

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Abstract

Treatment of primary cultures of rat ovarian dispersates with IL-1β results in morphologic and cytotoxic changes, thought to reflect tissue remodeling events associated with ovulation. We examined the role that the free radical nitric oxide plays in this process and report that IL-1β induces expression of the inducible isoform of nitric oxide synthase in ovarian cells as demonstrated by immunoprecipitation. We show that IL-1β treatment results in the formation of nitric oxide (as measured by accumulation of nitrite and cGMP) in both a time- and concentration-dependent manner that is prevented by aminoguanidine, a selective inhibitor of the inducible isoform of nitric oxide synthase. Aminoguanidine also inhibits IL-1-induced ovarian cellular cytotoxicity. These results suggest that nitric oxide is an important mediator of cell death and may act as a physiologically significant mediator of tissue remodeling events that occur in vivo during the ovulatory process. (*J. Clin. Invest.* 1993. 92:3053–3056.) Key words: cytokines • aminoguanidine • nitric oxide synthase • interleukin-1 • ovulation

Introduction

Ovulation, the process by which a mature ovum is generated and released, has been described as an orderly sequence of events reminiscent of an acute inflammatory response (1). Indeed, recent studies have pointed to the macrophage and its secretory products as potential in situ regulators of ovarian function (2). Specifically, IL-1-like activity has been demonstrated in follicular fluid (3, 4), and it appears that acquisition of intraovarian IL-1 synthetic capacity is gonadotropin dependent (2), pointing to an intermediary role for IL-1 in the ovulatory cascade. The perception that this cascade involves tissue remodeling and cell death, along with the observation that treatment of primary rat ovarian cell cultures with IL-1 produces marked time-dependent morphologic changes, including irreversible detachment and cell death (5), places IL-1 and the macrophage at the center of an incompletely described intraovarian network regulating follicular development and rupture.

Because of previous suggestions that IL-1-induced cytotoxicity in primary cultures of ovarian dispersates may be indirect and effected via secretion of soluble mediators (5), we hypothesized that the highly reactive free radical, nitric oxide ($NO^{\bullet}$),[1] whose diverse roles include regulation of vascular resistance and blood pressure, signal transduction, tumor cell killing, and resistance to infection (6–8), might be a critical effector molecule of IL-1-induced cell death in ovarian cell culture.

Methods

Reagents. Incubation medium was serum free CMRL-1066 (Gibco, Grand Island, NY) with glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). Collagenase type P and DNase were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Aminoguanidine hemi-sulfate salt, aprotinin, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO), and human recombinant IL-1β (10^9 U/mg) was obtained from Cistron Biotechnology, Inc. (Pine Brook, NJ).

Tissue culture preparation. Ovarian cells were isolated from immature (21–25 d-old) Sprague-Dawley rats (Sasco, Inc., O'Fallon, MO) and dispersed as described previously (9). Briefly, ovaries were harvested, cut into pieces, washed, and incubated in a collagenase-DNase solution of incubation media with 4 mg/ml collagenase and 10 μg/ml DNase for 2 h at 37°C. Successively smaller diameter Pasteur pipettes were then used to triturate the tissues, creating a cellular dispersate made up of a heterogeneous population of ovarian cells, including steroid secreting granulosa, theca, and interstitial cells, and resident ovarian macrophages (10, 11). Cells were washed twice before plating, as indicated, at either 2×10^5 cells/well in plastic flat-bottomed 96-well microtiter plates (for nitrite and lactic dehydrogenase [LDH] determinations) or 5×10^5 cells/well in 24-well plates (for cGMP determinations). Cells were incubated under an atmosphere of 95% air/5% CO_2 at 37°C. Supernatants were harvested at indicated times for analysis.

Nitrite determination. Nitrite determinations were made on 50-μl aliquots of culture supernatant mixed with 50 μl of the Griess reagent (12) and the absorbance at 540 nM was measured by Flow ELISA Reader (Flow Laboratories, Inc., McLean, VA).

Measurement of cGMP levels. cGMP accumulation was determined on ovarian dispersates incubated for 24 and 48 h as described above, followed by an additional 30-min incubation in fresh media

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Received for publication 27 April 1993 and in revised form 30 August 1993.

J. Clin. Invest.

0021-9738/93/12/3053/04 $2.00
Volume 92, December 1993, 3053–3056

1. *Abbreviations used in this paper:* AG, aminoguanidine; iNOS, inducible nitric oxide synthase; LDH, lactic dehydrogenase; $NO^{\bullet}$, nitric oxide.

containing 1 mM isobutyl-methylxanthine. Cells were isolated by centrifugation and cGMP was measured by radioimmunoassay as described previously (13).

Quantitation of cell death. Cell death was quantitated as described (14) by measurement of release of the soluble cytoplasmic enzyme LDH into culture supernatant. LDH content in 100-μl aliquots of cell-free supernatant was measured spectrophotometrically at 340 nM using NADH and sodium pyruvate as substrates. Cytotoxicity was calculated as:

$$\% \text{ cell death} = \frac{LDH_{sample} - LDH_{control}}{LDH_{total} - LDH_{control}} \times 100$$

where total LDH activity (LDH_{total}) was determined from the supernatant of a sample treated with 0.1% Triton X-100 for 30 min.

Immunoprecipitation of cytokine inducible NO• synthase (iNOS) from ovarian cells treated with IL-1β. Polyclonal antibodies were prepared against a peptide derived from the COOH-terminal 27 amino acids of mouse macrophage iNOS. Isolated ovarian cells (1×10^5/500 μl CMRL tissue culture media) were cultured overnight in a 24-well microtiter plate at 37°C under an atmosphere of 95% air and 5% CO_2. Cells were then washed three times with 1-ml portions of MEM methionine deficient media (9 parts methionine free MEM/1 part MEM containing methionine), and suspended in 400 μl of this combination of media. The cells were cultured for 18 h with or without IL-1β (10 U/ml) in the presence or absence of actinomycin D (1 μM). 300 μCi of [^{35}S]methionine (1,164 Ci/mmol, *Trans* ^{35}S-label, ICN Biochemicals, Inc., Irvine, CA) was added and the cells were cultured for 24 additional h. The media were removed and nitrite was determined as described above. The cells were washed three times with PBS, and then 0.5 ml of lysis buffer (0.1% Triton X-100, 0.1% BSA, 100 μg/ml aprotinin, 100 μg/ml leupeptin, 10 mM PMSF, and 1 mM iodoacetamide) was added and the cells were incubated at 4°C for 1 h. iNOS was immunoprecipitated from the lysed cells as described previously (15) using iNOS polyclonal antiserum at a 1:750 dilution. Immunoprecipitated samples were separated by SDS discontinuous polyacrylamide gel electrophoresis using an 8% gel (16).

Results

Effect of IL-1β on nitrite formation and cGMP accumulation in ovarian cell culture. Fig. 1 *a* demonstrates that 10 U/ml IL-1β induces the formation of nitrite (a stable oxidative metabolite of NO•) by ovarian cells following both 24- and 48-h incubations with IL-1β. Nitrite formation is blocked by nitric oxide synthase inhibitors aminoguanidine (17, 18) and N^G-monomethyl-L arginine (data not shown). Similarly, IL-1β treatment results in a fivefold increase in cellular cGMP at 24 h and a threefold increase at 48 h (Fig. 1 *b*). Aminoguanidine blocks cGMP formation at both time points. cGMP accumulation provides a sensitive index of NO• production because of the ability of NO• to directly activate guanylate cyclase (6).

Time and concentration dependence of IL-1β-induced nitrite formation by ovarian cell dispersates. The time course of IL-1β induction of ovarian cell nitrite formation is shown in Fig. 2 *a*. Treatment of ovarian cells with 10 U/ml IL-1β induces a linear increase in the formation of nitrite from 24 to 96 h. Aminoguanidine prevents the formation of nitrite at all time points examined. The concentration dependence of IL-1β-induced nitrite formation is shown in Fig. 2 *b*. IL-1β induces nitrite formation at a concentration of 10 U/ml, and nitrite production is substantially increased if cells are treated with 50 U/ml IL-1β; however, nitrite formation is not observed at concentrations of 1 U/ml or lower. Aminoguanidine (0.5 mM) completely blocks nitrite formation by ovarian cells treated with 50 U/ml IL-1β.

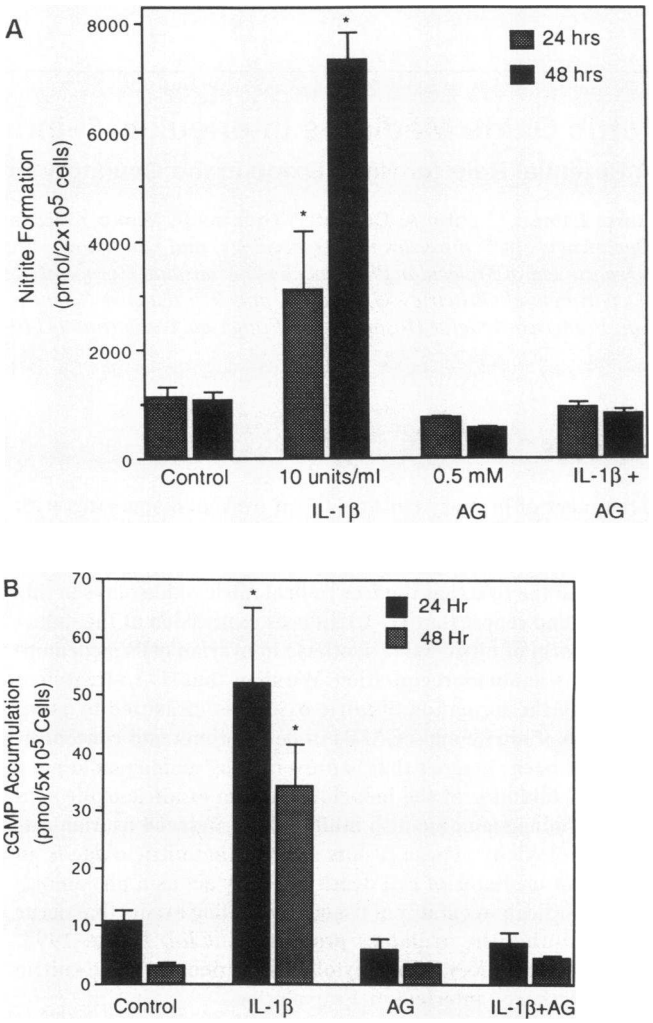

Figure 1. Effect of IL-1β on nitrite and cGMP formation by ovarian cells. The formation of nitrite (*A*) and the cellular accumulation of cGMP (*B*) by ovarian cells were determined following culture for 24 and 48 h in the presence or absence of 10 U/ml human recombinant IL-1β or 0.5 mM aminoguanidine (*AG*). Results are the mean±SEM of three independent experiments containing three replicates per condition.

Inhibition of NO• synthesis attenuates IL-1-induced ovarian cellular cytotoxicity. Several reports have identified NO• as the mediator of IL-1β induced cellular destruction in other experimental systems where induction of cytotoxicity generally requires protein synthesis (18–20). We studied the relationship between ovarian NO• production and IL-1-induced cytotoxicity by examining the ability of aminoguanidine to prevent IL-1β-induced ovarian cell death. Fig. 3 demonstrates that treatment of primary ovarian cell cultures for 96 h with 10 U/ml IL-1β results in the death of ~ 60% of cultured cells as measured by demise-dependent cellular release of LDH. This effect is significantly attenuated by inhibition of NO• synthesis in IL-1β-treated cultures using 0.5 mM aminoguanidine (IL-1β vs. IL-1β + aminoguanidine, $P < 0.01$). Treatment of ovarian cells with aminoguanidine alone, in the absence of IL-1β, does not effect cell viability. During the time period studied, no changes in either absolute or relative values of LDH release are observed in untreated control ovarian dispersates.

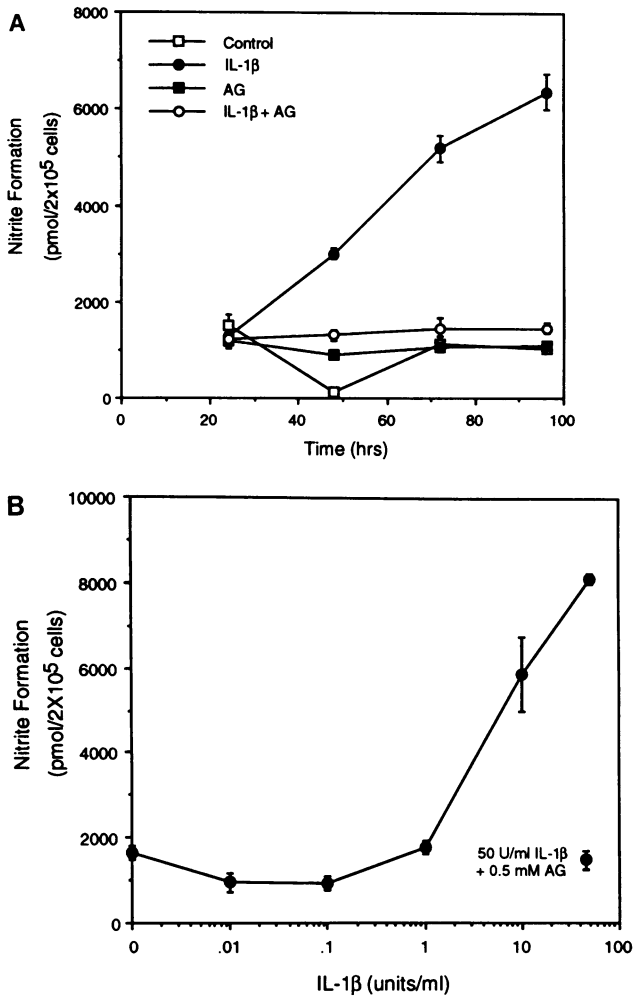

Figure 2. Time and concentration dependence of IL-1β-induced NO$^{\bullet}$ formation by ovarian dispersates. (*A*) Isolated ovarian cells (2×10^5 cells/100 μl) were cultured for the indicated periods of time in the presence or absence of 10 U/ml IL-1β or 0.5 mM aminoguanidine (*AG*) as indicated. (*B*) Isolated ovarian cells were cultured for 96 h in the presence of increasing concentrations of IL-1β as indicated. Results are the mean±SEM of three individual experiments containing three replicates per condition.

Identification of iNOS in the rat ovary by immunoprecipitation. Polyclonal antibodies specific for the COOH-terminal 27 amino acids of mouse macrophage iNOS were used to immunoprecipitate iNOS from ovarian cells treated with IL-1β. Fig. 4, lane *2* shows that treatment of ovarian cells with 10 U/ml IL-1 results in the expression of an immunoprecipitable protein with a size of 130 kD. The expression of this protein is not observed in untreated ovarian cells (lane *1*), and 1 μM actinomycin D (lane *3*) inhibits IL-1β-induced expression of this protein. The peptide used to prepare iNOS antiserum also prevents immunoprecipitation of iNOS induced by treatment of ovarian cells with IL-1β (lane *4*). This result, coupled with the inability of preimmune serum to immunoprecipitate iNOS from induced macrophages (our unpublished observation) demonstrates the specificity of the antiserum for the 130-kD protein. A threefold increase in the level of nitrite produced (control, 4.3±0.2 pmol/2×10^4 cells; IL-1, 14.5±0.25 pmol/2×10^4 cells) was found in IL-1-treated wells, and actinomycin D inhibited IL-1β-induced formation of nitrite (3.6±0.4 pmol/2×10^4 cells).

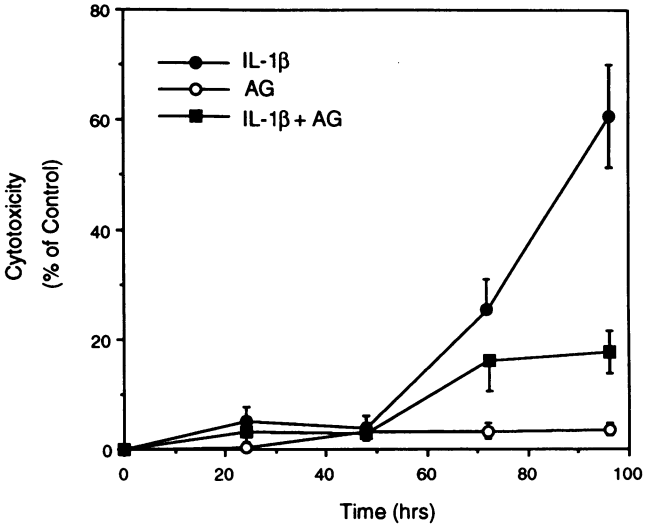

Figure 3. Effects of IL-1β and aminoguanidine (*AG*) on ovarian cell viability. Isolated ovarian cells were cultured for the indicated periods of time in the presence or absence of 10 U/ml IL-1β or 0.5 mM AG. Following culture, supernatants were removed and cell death was determined by the release of lactate dehydrogenase (LDH) into the culture medium. Results are the mean±SEM of three individual experiments containing three replicates per condition. $P < 0.01$ for IL-1 vs. IL-1 + AG at 72 and 96 h as determined by Student's unpaired *t* test.

Discussion

Our data demonstrate that IL-1β actively induces NO$^{\bullet}$ synthesis in the ovary via induction of expression of a 130-kD protein immunoprecipitated with iNOS antiserum. Moreover, the findings presented here establish that NO$^{\bullet}$ is a critical mediator of IL-1-induced cytotoxicity in ovarian cell culture. It is also likely that ovarian production of NO$^{\bullet}$ is of great importance in vivo. In addition to a cytotoxic effector role in tissue remodeling and ovulation, the molecule could also be a mediator (along with histamines and prostaglandins) of the vasodilation

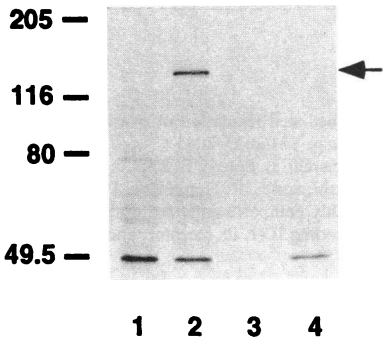

Figure 4. Immunoprecipitation of cytokine iNOS from ovarian cells treated with IL-1β. Ovarian cells were metabolically labeled under the following conditions: lane *1*, untreated control; lanes *2* and *4*, 10 U/ml IL-1β; lane *3*, 10 U/ml IL-1β and 1 μM actinomycin D. After culture, the cells were lysed and a 130-kD protein was immunoprecipitated by polyclonal antiserum prepared against mouse macrophage inducible nitric oxide synthase. In lane *4*, 0.5 μg of the peptide antigen used to prepare the polyclonal antiserum was added during the immunoprecipitation reaction. Samples were separated by 8% SDS polyacrylamide gel electrophoresis and proteins were visualized by fluorography. Results are representative of two independent experiments.

and resulting follicular hyperemia associated with early preovulatory events (21) such as follicular recruitment and selection. Although less well understood in terms of cytokine involvement, the process of follicular atresia would also seem to require the participation of short-lived cytotoxic mediators such as the $NO^{\bullet}$ molecule.

Presently, the cellular source of ovarian $NO^{\bullet}$ synthesis is not known. Although a likely candidate is the ovarian macrophage, it is important to note that IL-1 has not been demonstrated to induce $NO^{\bullet}$ synthesis by the macrophage (22). It is quite possible that other cells, particularly of nonhematopoietic origin, are capable of expressing iNOS, as has been shown in the pancreatic islet β-cell (18) and in endothelial cells (19). The observations that neither purified granulosa or theca cell cultures alone exhibit the IL-1 cytotoxic effect, and that the effect can be recovered in co-culture of the two purified populations, suggests not only that IL-1 acts via induction of a soluble mediator (5), which we have identified as $NO^{\bullet}$, but also that the cytotoxic effect requires specific interactions between the two cell populations to induce the expression of $NO^{\bullet}$ synthase. Regulation of $NO^{\bullet}$ synthesis in these two cell populations is currently under active investigation.

Other reports have identified $NO^{\bullet}$ production in reproductive tissues. $NO^{\bullet}$ release by noncholinergic, nonadrenergic neurons has been shown to be the major, if not only, neuronal mediator of smooth muscle relaxation of the corpus cavernosum and thus penile erection (23, 24). Our description of $NO^{\bullet}$ production in another reproductive tissue, under very different conditions, mediating a fundamentally different process, points to intimate involvement of this "Janus-faced" molecule (25) at many disparate points in the reproductive process.

Acknowledgments

We thank Dr. E. R. Unanue, Department of Pathology, and Drs. J. S. Schreiber and L. Olsen, Department of Obstetrics and Gynecology, all at Washington University, St. Louis MO for their generous support and encouragement of this work. We are also grateful to Connie Marshall for providing expert technical assistance.

This work was supported by National Institutes of Health grants DK-06181 (to M. L. McDaniel), F32-DK-08748 (to J. A. Corbett) and Public Health Reproductive Scientist Development Award HD 00849-04 (to K. P. Beckerman).

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