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NITRIC OXIDE PRODUCTION BY SERTOLI CELLS IN RESPONSE TO CYTOKINES AND LIPOPOLYSACCHARIDE

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SUMMARY: Nitric oxide (NO) is formed from L-arginine residues by nitric oxide synthase (NO Synthase) in many types of cells and acts as an intercellular messenger in several physiological systems. In the present study, we demonstrate that a combination (CL) of interleukin-1α, interferon γ, tumor necrosis factor α and lipopolysaccharide induces nitrite (NO_2^-) production in cultured rat Sertoli cells. This biosynthesis of NO_2^- requires a lag time period of 18 hr and then increases for at least 96 hr; it is prevented by two NO Synthase inhibitors, N^G-monomethyl-L-arginine and aminoguanidine. Northern blot analysis shows the induction of a macrophage-like NO Synthase mRNA synthesis in Sertoli cells cultured for a minimum of 6 hr in the presence of CL, with maximal levels after 12 to 30 hr of incubation. These results indicate for the first time that cultured rat Sertoli cells express an inducible NO Synthase isoform in response to a combination of cytokines and lipopolysaccharide.

The free radical gas nitric oxide (NO) has now been shown to be an important short-lived mediator formed in a variety of cell types. It participates in numerous physiological processes including neurotransmission, vascular relaxation, platelet aggregation and immune defense (for review, see 1).

NO is synthesized from the guanidino nitrogen of L-arginine by at least three isoforms of NO synthase (1). The neuronal NO Synthase (nNO Synthase) and the endothelial NO Synthase (eNO Synthase) are constitutive and calcium / calmodulin dependent (1-3), whereas the macrophage NO Synthase is an inducible calcium independent isoform (iNO Synthase) (1, 3). In many cell types including macrophages (4), hepatocytes (5), endothelial (6) and smooth muscle cells (7), cytokines and endotoxins like lipopolysaccharide (LPS) induce the

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Abbreviations: NO, nitric oxide; NO_2^-, nitrite; eNO Synthase, endothelial nitric oxide synthase; nNO Synthase, neuronal nitric oxide synthase; iNO Synthase, inducible nitric oxide synthase; IL-1α, interleukin-1α; IFNγ, interferon γ; TNFα, tumor necrosis factor α; LPS, lipopolysaccharide; L-NMMA, N^G-monomethyl-L-arginine; AG, aminoguanidine.

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expression of iNO Synthase, thus resulting in the production of a large amount of NO. Recently, in the testis, Sertoli cells and germ cells have been shown to produce several cytokines (8-12), some of them being induceable by LPS (8) and which probably participate in the spermatogenetic process.

In the present study, we report that a mixture of cytokines and LPS is able to stimulate NO production by Sertoli cells. Moreover, we demonstrate that, in the presence of these compounds, mRNA for macrophage-like iNO Synthase is induced in this testicular cell type.

MATERIALS AND METHODS

Material. Male Sprague-Dawley rats were purchased from Elevage Janvier (Le Genest, Saint Isle, France). Rat interferon γ (IFNγ) was a gift from Dr van der Meide (TNO Center, Rijswijk, Holland). Human recombinant tumor necrosis factor α (TNFα) was kindly supplied by Dr E Bosmans (Eurogenetics, Tessenderlo, Belgium). Human recombinant interleukin-1α (IL-1α) was from Sigma Chemical Co (St Louis, MO, USA). Griess reagent was purchased from Merck (Nogent sur Marne, France). Culture media and other reagents for cell cultures were obtained from Gibco BRL (Cergy Pontoise, France). Nylon membranes were purchased from NEN Dupont (Les Ulis, France), [α-^{32}P]dCTP was from Amersham Life Science (Les Ulis, France) and all other products were purchased from Sigma Chemical Co.

Sertoli cell isolation and culture conditions. Sertoli cells were prepared from 20-day-old Sprague-Dawley rats, as described by Skinner *et al.* (13), and cultured at 32°C in a humidified atmosphere of 5% CO_2-95% air in Ham's F12/DMEM medium (1:1 v/v) without phenol red, supplemented with gentamicin (50 μg/ml), insulin (10 μg/ml) and transferrin (5μg/ml). After 3-4 days of culture, the Sertoli cells were exposed to a hypotonic shock (20 mM Tris-HCl buffer solution, pH 7.4) for 2 min to remove contaminating germ cells (14). Under these conditions, the degree of purity of the Sertoli cell culture was ≥ 98%. On day 5 of culture, the cells were incubated for various periods of time in transferrin-free medium supplemented or not with cytokines (C: IL-1α (5 U/ml), TNFα (500 U/ml), IFNγ (100 U/ml)) and LPS (L: 10 μg/ml). The CL treatment was performed in the absence or presence of 1 mM N^G-monomethyl-L-arginine (L-NMMA) or 0.5 mM aminoguanidine (AG), two inhibitors of NO synthase (3, 15). The viability of the treated Sertoli cells was estimated by MTT staining (16). At the end of incubation, media were recovered, centrifuged and frozen at -80°C until tested for nitrite content. In parallel, cells were treated with guanidium thiocyanate for RNA extraction (17, 18) or frozen at -20°C for DNA content assay (19).

Measurement of nitrite. NO released by Sertoli cells was evaluated by assay of culture supernatants for nitrite (NO_2^-), a stable end product of NO oxidation (20), using the Griess reagent, as described elsewhere (21). Concentrations of NO_2^- in the samples were calculated by comparison with a standard curve obtained with $NaNO_2$ solutions prepared with culture medium.

Molecular probes. The iNO Synthase probe used was a ^{32}P-labeled *Not I*-digested cDNA insert from a mouse macrophage cDNA clone kindly provided by Dr Charles J. Lowenstein, Johns Hopkins University, Baltimore, USA (4). The corresponding 4 Kb insert was found to effectively identify the rat macrophage NO Synthase mRNA on cross-species hybridization (*see Results*).

RNA isolation and Northern blot analysis. Total RNA was extracted from cultured Sertoli cells with guanidium thiocyanate, followed by centrifugation in cesium chloride solution (17, 18). Every RNA sample had an OD260/OD280 ratio > 1.50. Thirty µg of total RNA were electrophoresed in a 2% formaldehyde - 1% agarose gel, then transferred to a nylon membrane and UV-crosslinked. The membranes were hybridized for 20 hr with the [α-^{32}P]dCTP cDNA probe labeled by random priming (1.5×10^6 cpm/ml), as previously described (22). mRNA from rat peritoneal macrophages stimulated or not with CL were used as a control for the detection of the iNO Synthase mRNA. Autoradiography was performed by exposure overnight to Kodak X-Omat film at -80°C, in the presence of intensifying screens. To correct for differences in RNA amount, blots were stripped and rehybridized with a radiolabeled cDNA probe for rat actin.

Statistical analysis Results are expressed as means ± SEM. Differences in mean were analysed using a paired one-tailed Student's t-test. Significance was defined as $P < 0.05$.

RESULTS

Effect of the CL on Sertoli cell nitrite formation. Fig.1 presents the time course of NO_2^- production by Sertoli cells cultured in control medium or in the presence of CL. NO_2^- accumulation in the control medium was very low over 96 hr and corresponded to a constant production rate of 3 ± 1 pmol/µg DNA/hr (n=5). In the presence of CL, after a lag time period of 12 hr, Sertoli cells released significantly higher amounts of NO_2^- from 18 to 96 hr of incubation, as compared to the control. In fact, between 18 to 48 hr, NO_2^- production corresponded to 15 to 36 pmol NO_2^- produced/µg DNA/hr and between 48 to 96 hr of incubation, it reached a rate of 80 ± 16 pmol NO_2^- produced/µg DNA/hr (n=5). Coincubation with L-arginine analogs, like L-NMMA or AG, clearly inhibited this CL-stimulated release of NO_2^- by cultured Sertoli cells (Fig. 2). This was not due to cytotoxicity, since L-NMMA or AG had no effect on cell viability, as determined by MTT test (data not shown).

Effect of CL treatment on iNO synthase mRNA levels. Total RNA was isolated from Sertoli cells at various times after exposure to CL and analyzed in Northern blot with a cDNA probe for iNO Synthase mRNA. The results presented in Figure 3 indicate that Sertoli cells incubated in control medium (U) faintly express iNO Synthase mRNA whereas, after 6, 12, 24 or 30 hr of incubation with CL, a strong single band of approximately 4 Kb was present. Similar size transcript was observed from CL-stimulated rat peritoneal macrophages RNA used as positive control (Fig. 3A). The densitometric analysis of the autoradiographic iNO Synthase mRNA signals, after normalization to the respective actin signal, shows that the level of iNO Synthase mRNA was stimulated after 6 hr incubation with CL, peaked after 12 hr and plateaued at maximal values, thereafter (Fig. 3B).

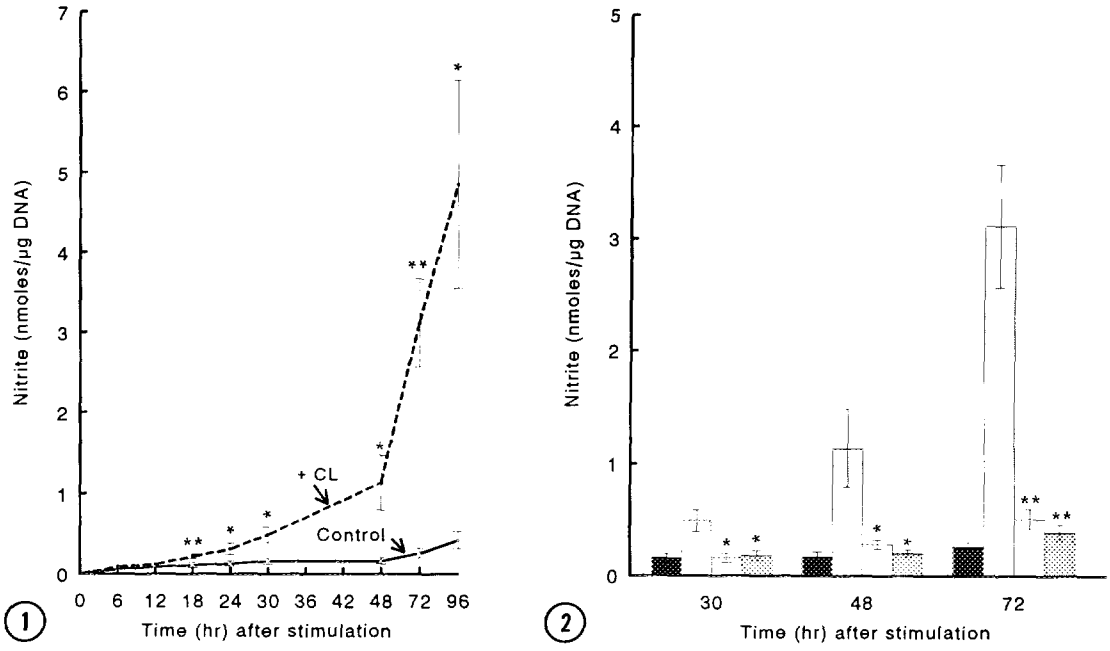

Figure 1. Time course of NO_2^- production by rat Sertoli cells. Cells were incubated for various periods of time in Ham' s F12/DMEM medium supplemented (- - - -) or not (———) with CL (C for: IL-1α (5 U/ml), TNFα (500 U/ml), IFNγ (100 U/ml) and L for LPS (10 µg/ml)).NO_2^- concentrations in culture media were determined as described in the Materials and Methods section. Results are means ± SEM for 5 separate experiments, each performed in triplicates, and are expressed as nmoles NO_2^- formed / µg DNA. * , $0.01 < P < 0.05$; ** , $0.001 < P < 0.01$, as compared to respective unstimulated values.

Figure 2. Inhibitory effect of two NO synthase inhibitors, N^G-monomethyl-L-arginine (L-NMMA) and aminoguanidine (AG), on NO_2^- production by stimulated Sertoli cells. Cells were incubated for various times in Ham's F12/DMEM medium alone (▩) or supplemented with CL (□), CL plus 1 mM L-NMMA (□) or CL plus 0.5 mM AG (▨). NO_2^- concentrations in culture media were determined as described in the Materials and Methods section. Results are means ± SEM for 5 separate experiments, each performed in triplicates and are expressed as nmoles NO_2^- formed / µg DNA. * , $0.01 < P < 0.05$; ** , $0.001 < P < 0.01$, as compared to respective stimulated values.

DISCUSSION

Several recent studies demonstrate that NO may be an inhibitory regulator of steroidogenesis (23, 24) and suggest its important role in male fertility regulation (25). However, to our knowledge, testicular NO biosynthesis has not yet been studied. In this study, we demonstrate that NO synthesis by cultured Sertoli cells, measured as NO_2^- production, can be highly stimulated, in the presence of a combination of IL-1α, TNFα, IFNγ and LPS, in a time-dependent manner. The strong inhibition of NO_2^- production by L-NMMA and AG, two L-arginine analogs, indicates that it is likely dependent upon a NO Synthase. Furthermore, the

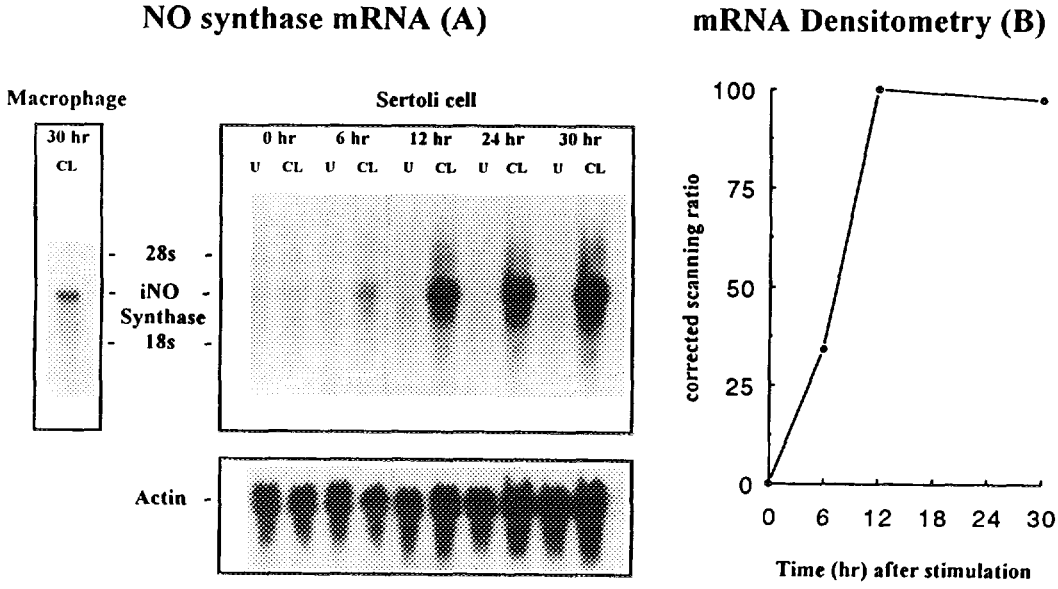

Figure 3. Time course of rat Sertoli cells iNO Synthase mRNA induction after CL exposure. Sertoli cells were cultured in Ham's F12/DMEM medium alone (unstimulated cells ; U), or supplemented with CL (CL) for the indicated time periods (A). Total cellular RNA was hybridized either to ^{32}P-labeled iNOS cDNA probe or to ^{32}P-labeled actin cDNA probe. As a positive control, Northern analysis of RNA from 30 hr-CL stimulated rat peritoneal macrophages was performed under the same conditions. mRNA signals were quantitated by scanning densitometry and corrected relative to actin signal. The corrected density was then plotted as a percentage of maximal stimulation (B). Blots shown are representative of 3 separate experiments.

lag period of several hours required for CL induction of Sertoli cell NO Synthase activity, associated with a sustained NO_2^- production for several days, are characteristic of the action of a macrophage like iNO Synthase. This hypothesis is confirmed by the fact that Sertoli cells cultured with CL for at least 6 hr presented high levels of a 4 Kb mRNA which hybridized with a cDNA for inducible macrophage NO Synthase.

Recent reports demonstrate that cytokines such as IL-1α (8,9), TNFα (10), IFNγ (11), and IL-6 (12) are produced within seminiferous tubules and might play important roles in the regulation of spermatogenesis (26). Our results show that Sertoli cell NO Synthase can be induced, *in vitro*, by a combination of cytokines plus LPS and therefore suggest that NO may be involved in the mediation of the cytokine effects within the testis.

At present, the physiological role of NO biosynthesis by Sertoli cells is unknown. However, since NO has been demonstrated to be a diffusible intercellular messenger (27, 28) and probably a cell growth regulator (1, 29, 30), it is tempting to hypothesize that this molecule is involved in the local control of spermatogenesis. Furthermore, NO production in different

types of cells has been shown to be cytotoxic against bacteria and virus (1). Therefore, NO generated by Sertoli cells may be involved in the testicular host defense system. Further experiments are needed to define the cell targets for NO and the precise roles of this molecule within the testis.

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