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# Interleukin-1β Regulates Nitric Oxide Production and γ-Glutamyl Transpeptidase Activity in Sertoli Cells

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**ABSTRACT:** Several cytokines have been involved in the regulation of Sertoli cell function. Further investigations are required to elucidate the role of interleukin-1β (IL1β) in Sertoli cell physiology. Twenty-day-old rat Sertoli cell cultures were used to investigate a possible role of IL1β in the regulation of γ-glutamyl transpeptidase (γGTP) and to elucidate the signaling pathway utilized by this cytokine. γGTP is a membrane-bound enzyme that has been involved in amino acid transport across the plasma membrane and in protection from oxidative stress through its importance in the regulation of glutathione levels. Previous studies suggested that IL1β stimulates NO biosynthesis in other cell types. Therefore, we investigated whether IL1β modified the level of nitrite, a stable metabolite of NO, in Sertoli cells. Dose–response curves to IL1β for γGTP activity and nitrite production were observed. The increments observed in γGTP activity and nitrite production were partially and completely blocked by simultaneous treatment with the NO synthase inhibitor aminoguanidine. Treatment of Sertoli cell cultures with the NO donors sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine resulted in an increase in γGTP activity. The presence of neural, endothelial, and inducible isoforms of NO synthase (NOS) was investigated by a immunohistochemical technique using specific antibodies. The 2 constitutive isoforms were present under basal conditions, and the inducible protein appeared in IL1β-treated cultures. Finally, translocation of NF-κB p65 subunit to the nucleus in IL1β-treated cultures was observed. These findings suggest that the action of IL1β on Sertoli cell γGTP activity is partially mediated via activation of NF-κB and increments in iNOS and cellular production of NO.

Key words: Testis, cytokines, nitric oxide synthase, NF-κB, signal transduction.

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A growing body of evidence suggests that cytokines may belong to the large set of intratesticular regulators that provide the fine-tuning of cellular processes implicated in the maintenance of spermatogenesis (Gnessi et al, 1997). The actions of interleukin 1 (IL1), comprising IL1α and IL1β, on the regulation of Sertoli cell function have been previously studied. IL1α regulates IL6 secretion (Syed et al, 1995), plasminogen activator activity (Sigillo et al, 1998), expression of mRNA for IL1α and IL1 receptor type I (Wang et al, 1998), and sertolin mRNA levels (Mruk and Cheng, 1999). Moreover, IL1β regulates estradiol production (Khan and Nieschlag, 1991), transferrin secretion (Hoeben et al, 1996b), lactate production (Nehar et al, 1998), gelatinase A secretion (Hoeben et al, 1996a), and IL6 expression (Okuda et al, 1995). With few exceptions, these reports have not investigated the signal transduction pathways utilized by these cytokines. IL1β, primarily an inflammatory cytokine, is produced by interstitial macrophages and Leydig cells (Lin et al, 1993; Hayes et al, 1996) in the testis, and it has been shown that IL1 receptors type I and II mRNAs are constitutively expressed in Sertoli cell cultures (Gomez et al, 1997).

Several signal transduction pathways have been shown to be activated in response to IL1β in various cell types (Bankers-Fulbright et al, 1996). Among them, nitric oxide (NO) has been identified as a mediator of IL1β action in a wide variety of tissues, including vascular smooth muscle cells (Beasley and Eldridge, 1994), hepatocytes (Wang et al, 1998), pancreatic islets (Bergmann et al, 1992), and Leydig (Tatsumi et al, 1997) and granulosa cells (Chun et al, 1995). The free radical NO is synthesized via the oxidation of a guanidine-nitrogen atom of L-arginine in a process catalyzed by the enzyme nitric oxide synthase (NOS; Ignarro, 1990; Moncada et al, 1991). Two major types of NOS isoenzymes with distinct regulatory properties have been described: constitutive calcium-dependent enzymes, originally found in endothelial cells (eNOS; Lamas et al, 1992) and in certain populations of neurons (nNOS; Bredt and Snyder, 1990), and an inducible calcium-independent isoform originally found in macrophages (iNOS; Xie et al, 1992). In several cell lines,

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cytokines increase iNOS expression by nuclear translocation of the transcription factor NF-κB (Taylor et al, 1998). It is notable that iNOS expression in response to a combination of different cytokines has been observed in Sertoli cells (Bauche et al, 1998). However, the role of IL1β in the expression of iNOS in Sertoli cells has not been investigated yet.

γGTP is a membrane-bound enzyme that is widely distributed in mammalian tissues. It has been suggested that among other functions, this enzyme acts as a transport system for amino acids across the plasma membrane (Tate and Meister, 1981). In addition, γGTP activity, indirectly and through the regulation of glutathion levels, seems to be involved in protection from oxidative stress (Hanigan and Ricketts, 1993).

In the present study, we evaluated the effect of IL1β on the regulation of Sertoli cell γGTP activity and the possible role of NO as a mediator of IL1β action. In addition, we tested whether NF-κB translocation and iNOS expression might be involved in IL1β action on Sertoli cells.

## Materials and Methods

### Materials

Tissue culture media were purchased from Grand Island Biological Co (Grand Island, NY). Human recombinant IL1α and β from Sigma Chemical Co (St Louis, Mo) were used. All other drugs and reagents were also purchased from Sigma Chemical Co.

### Sertoli Cell Isolation and Culture

Sertoli cells from 20-day-old Sprague-Dawley rats were isolated as previously described (Meroni et al, 1997). Briefly, decapsulated testes were digested with 0.1% collagenase and 0.06% soybean trypsin inhibitor in Hanks balanced salt solution for 5 minutes at room temperature. Seminiferous tubules were saved, cut, and submitted to 1 M glycine–2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 minutes at room temperature to remove germinal cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium consisting of a 1:1 mixture of Ham F-12 and Dulbecco modified Eagle medium, supplemented with HEPES 20 mM, 100 IU/mL penicillin, 2.5 μg/mL amphotericin B, 1.2 mg/mL sodium bicarbonate, 10 μg/mL transferrin, 5 μg/mL insulin, and 5 μg/mL vitamin E. Sertoli cells were cultured in 24 multiwell plates (10 μg DNA per well) at 34°C in a mixture of 5% $CO_2$:95% air. Cells were allowed to attach for 48 hours. Treatments started on day 3 and were performed in culture medium without the addition of insulin.

Purity of Sertoli cells reached 95% after 5 days in culture as examined by phase–contrast microscopy. No myoid cell contamination was revealed when an immunoperoxidase technique was applied to Sertoli cell cultures using a specific antiserum to alpha-smooth muscle actin. Remaining cell contaminants were of germ cell origin.

### Culture Conditions

Sertoli cell cultures were stimulated with IL1β or the NO donors on day 3, and γGTP activity was determined on the cells harvested on day 6. To evaluate nitrite production, Sertoli cell cultures were stimulated with IL1β on day 5, in medium without phenol red dye, and media were collected 24 hours later for nitrite assay. Aminoguanidine (AG) was used to inhibit NOS activity. This drug was added simultaneously with IL1β in both cases.

Cell viability at the end of the culture period was evaluated by a trypan-blue exclusion test. Under all experimental conditions tested, cell viability was higher than 95%.

### γ-Glutamyl Transpeptidase Assay

γGTP activity was assayed by the method of Orlowsky and Meister (1963), using L-γ-glutamyl *p*-nitroanilide as substrate and glycylglycine as the acceptor molecule. Sertoli cell monolayers were disrupted by ultrasonic irradiation in 0.5 mL reaction buffer (0.1 M Tris buffer, 0.01 M $MgCl_2$, and 0.02 M glycylglycine; pH 9). Adequate aliquots for DNA determinations were saved, and 5 mM substrate (L-γ-glutamyl *p*-nitroanilide) was added to the remaining material. The reaction was allowed to proceed for 120 minutes at 34°C, and the enzymatic reaction was stopped by addition of acetic acid up to a 1 M concentration. Samples were then centrifuged, and absorbances were determined in a spectrophotometer at 410 nm. Values were compared against a standard curve with increasing concentrations of *p*-nitroaniline. DNA was determined by the method of Labarca and Paigen (1980), and the results were expressed on a per-microgram-DNA basis.

### Nitrite Assay

To determine the amount of NO released by Sertoli cells, the conditioned media were assayed for nitrite, a stable end product of NO oxidation, by using the Griess reagent (1:1 mixture of 1.32% sulfanilamide in 60% acetic acid with 0.1% N-1-naphthyl-ethylenediamine-HCl; Green et al, 1982). Concentrations of nitrite in the samples were calculated by comparison with a standard curve obtained using $NaNO_2$ solutions prepared with culture medium. Nitrite concentration in the culture medium was also expressed per microgram DNA.

### Immunohistochemistry for NOS and NF-κB

Immunohistochemical detection of NOS isoenzymes was performed on Sertoli cells cultured under basal conditions. Inducible NOS was also determined in Sertoli cells that had received stimulation with IL1β for 3 days. For NF-κB immunohistochemistry, 6-day-old monolayers were incubated with IL1β for 2 hours. Paired nonstimulated cultures were also immunostained. At the end of the experiments (day 6), monolayers were washed in phosphate-buffered saline (PBS) and fixed for 3 hours in a mixture containing paraformaldehyde and picric acid in phosphate buffer (Suburo et al, 1995). After washing in PBS, monolayers were treated for 30 minutes with 0.3% hydrogen peroxide to quench endogenous peroxidase activity and were permeabil-

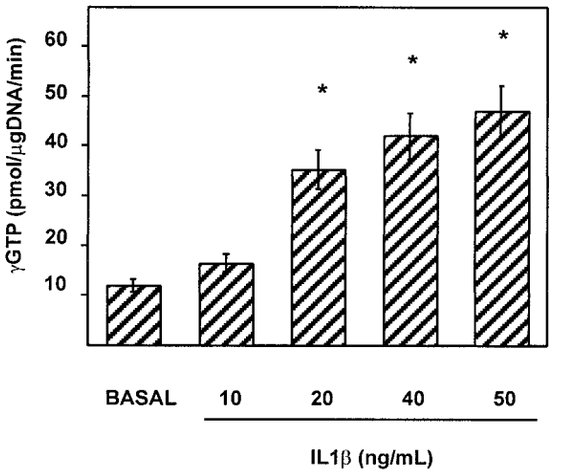


Figure 1. Effect of interleukin 1β (IL1β) on γ-glutamyl transpeptidase (γGTP) activity. Sertoli cells were cultured for 3 days under basal conditions (BASAL) or with increasing concentrations of IL1β. γGTP was evaluated on the sixth day. Values are expressed as mean ± SD of triplicate incubations in 1 representative experiment out of 3. *$P < .01$ vs basal.

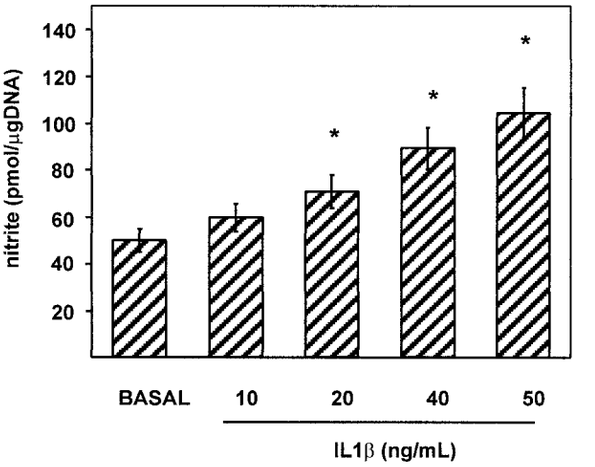


Figure 2. Effect of IL1β on nitrite production. Sertoli cells were cultured for 4 days in control medium. On the fifth day, cells were stimulated with increasing concentrations of IL1β. Twenty-four hours later, media were collected, and nitrite was determined. Values are expressed as mean ± SD of triplicate incubations in 1 representative experiment out of 3. *$P < .01$ vs BASAL.

ized with 0.3% Triton X100 in PBS. The monolayers were incubated overnight at 4°C with the primary antibody. Antibody binding was detected with biotinylated antiserum and avidin-biotin-peroxidase complex (Vectastain Elite, Vector Labs, Burlingame, Calif). Peroxidase activity was revealed through the use of diaminobenzidine with nickel enhancement. Immunohistochemical controls were made by substituting PBS for the primary antibody. Further negative controls using nonimmune mouse serum (DAKO Laboratories, Carpenteria, Calif) and mouse anti-human CD68 (DAKO Laboratories) were performed. The following antibodies were used: 1) nNOS monoclonal ab (Transduction Laboratories, Lexington, Ky), 2) ECNOS monoclonal ab (Transduction Laboratories), 3) iNOS goat polyclonal ab (Santa Cruz Biotechnology, Santa Cruz, Calif), and 4) NF-κB p65 goat polyclonal ab (Santa Cruz Biotechnology).

### *Statistical Analysis*

Statistical analysis was performed by analysis of variance, followed by Tukey's protected *t* test for comparison of data from multiple groups by using the GB-STAT version 4.0 statistical program (Dynamic Microsystems, Inc, Silver Spring, Md).

## *Results*

### *Effect of IL1β on γGTP Activity and Nitrite Production*

In order to evaluate a possible effect of IL1β on γGTP activity, Sertoli cells were stimulated with different doses of IL1β for 3 days. Figure 1 shows the dose-response effect for the cytokine. The lowest dose that produced a statistically significant stimulation of γGTP activity was 20 ng/mL.

In order to evaluate the effect of IL1β on NO production, Sertoli cell cultures were treated with different doses of IL1β for 24 hours, and a stable NO metabolite—nitrite—was assayed in the culture medium. Figure 2 shows the dose–response effect of IL1β on nitrite production. The lowest dose that produced a statistically significant increase in nitrite production was also 20 ng/mL. IL1α (30 U/mL) did not produce an increase in nitrite production (basal: 53.4 ± 3.2 pmol/μg DNA; IL1α: 55.1 ± 2.7 pmol/μg DNA) while increasing transferrin secretion (data not shown).

### *Participation of NO in the Regulation of γGTP Activity by IL1β*

To study the possible involvement of a NO-dependent pathway in the regulation of γGTP activity, cells were incubated with different doses of the NO donors sodium nitroprusside (NPS) and *S*-nitroso-*N*-acetyl penicillamine (SNAP) for 3 days. Figure 3 shows that both drugs stimulated γGTP activity.

Next, we analyzed whether NO production was involved in the stimulatory effect that IL1β exerted on γGTP activity. For this purpose, cultures were stimulated with IL1β in the presence or absence of a NOS activity inhibitor—namely, aminoguanidine (AG). Figure 4 (upper panel) shows that the drug completely inhibited the stimulation of nitrite production induced by IL1β. Figure 4 (lower panel) shows that stimulation of γGTP activity by IL1β was partially inhibited when AG was present in the culture medium.

### *Immunohistochemistry for NOS Isoforms in Sertoli Cell Cultures*

Sertoli cell monolayers were immunohistochemically stained using specific antibodies to eNOS, nNOS, and

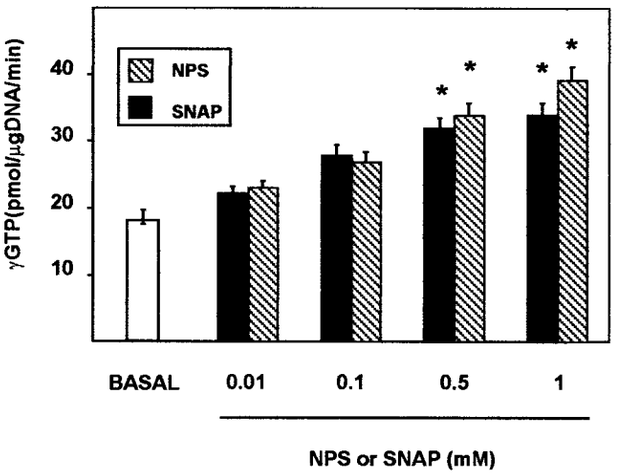


Figure 3. Effect of nitric oxide (NO) donors on γGTP activity. Sertoli cells were cultured for 3 days under basal conditions (BASAL) or with increasing concentrations of the NO donors sodium nitroprusside (NPS) and *S*-nitroso-*N*-acetyl penicillamine (SNAP). γGTP was evaluated on the sixth day. Values are expressed as mean ± SD of triplicate incubations in 1 representative experiment out of 3. *$P < .01$ vs basal.

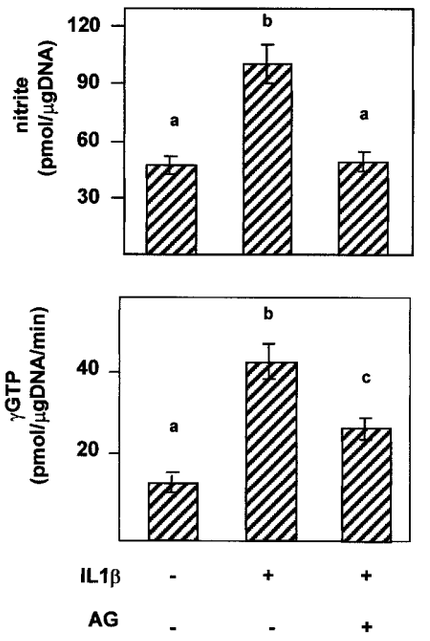


Figure 4. Effect of aminoguanidine (AG) on IL1β-stimulated γGTP activity and nitrite production. Sertoli cells were cultured without stimuli or in the presence of IL1β (50 ng/mL) with or without the addition of aminoguanidine (1 mM). Values are expressed as mean ± SD of triplicate incubations in 1 representative experiment out of 3. Different superscripts indicate statistically significant differences ($P < .01$).

iNOS. Positive staining for the 2 constitutive isoforms was observed (Figure 5A and B). iNOS was not immunohistochemically detected in nontreated cultures (Figure 5C). By contrast, cells treated for 3 days with IL1β (50 ng/mL) showed iNOS immunostaining (Figure 5D).

### *NF-κB Nuclear Translocation Induced by IL1β*

To analyze whether IL1β was able to produce the nuclear translocation of NF-κB in rat Sertoli cells, immunohistochemical staining of p65 NF-κB in basal and IL1β-stimulated cultures was performed. In nonstimulated cells, p65 NF-κB immunoreactivity had a distinct cytoplasmic localization (Figure 6A). By contrast, in IL1β-stimulated cells, a strong p65 NF-κB immunoreactivity appeared in cell nuclei. A faint cytoplasmic immunostaining was also observed (Figure 6B).

## *Discussion*

It has been shown that IL1β is capable of producing a wide variety of effects on many different cell types. Nearly every known signal transduction pathway has been reported to be activated in response to IL1β. However, the significance of many of these signaling events is unclear because of the use of different and sometimes unique cell lines in studying IL1β-initiated signal transduction. Complicating matters further, there is a lack of association in many studies between identified IL1β-induced signals and subsequent biological responses. Sertoli cell function is controlled by hormones such as FSH and testosterone and also by various paracrine factors, which include cytokines such as IL1β (Jegou, 1993).

The ability of Sertoli cells to respond to a combination of certain cytokines by increasing nitrite production has already been shown (Stephan et al, 1995). However, no data were available on the ability of IL1β in particular to increase NO production in Sertoli cells. Our results show that IL1β increases both the levels of a stable metabolite of NO and γGTP activity in Sertoli cells. Bauche et al (1998) have shown that treatment with 5 U/mL human recombinant IL1α does not produce an increment of nitrite production. In agreement with the latter results, we have not observed an increment of nitrite production with 30 U/mL human recombinant IL1α. The ability of IL1β to stimulate nitrite production was somehow unexpected, as IL1β and IL1α utilize the same receptor and elicit similar biological responses (Nehar et al, 1998). Different biological potencies have been observed when using cytokines from human origin on rat cells (Moynagh et al, 1993). Whether this variance in biological potency might be responsible for the results obtained remains unknown.

As for the possible relationship between NO production and stimulation of γGTP activity, we have observed that 2 different NO donors stimulate γGTP activity. The latter observations suggest that stimulation of γGTP activity by IL1β might be mediated by endogenous NO production. This hypothesis gains further support by the finding that

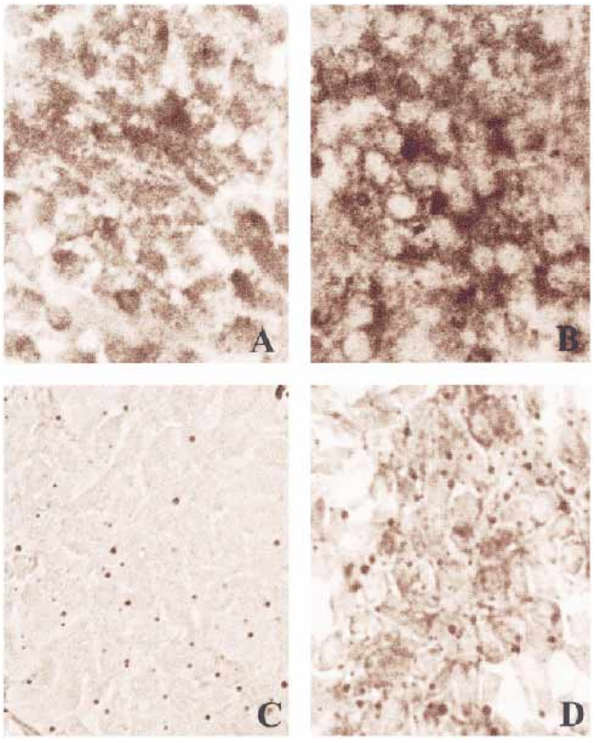


Figure 5. Nitric oxide synthase (NOS) isoforms in Sertoli cells. Sertoli cell monolayers were immunohistochemically stained using specific antibodies to neural, endothelial, and inducible NOS. Positive staining for the 2 constitutive isoforms was observed **(A, B)**. iNOS was not immunohistochemically detected in basal cultures **(C)**. By contrast, cells treated for 3 days with IL1β (50 ng/mL) showed iNOS immunostaining **(D)**. The immunostaining results shown are representative of 3 independent experiments (450×).

stimulation of γGTP activity by IL1β was partially blocked by the addition of aminoguanidine, an inhibitor of NO synthase.

NO is an important regulator of the reproductive system (Rosselli et al, 1998). In the testis, it has been indicated that endogenously produced NO as well as NO-releasing agents inhibit steroidogenesis in Leydig cells (Del Punta et al, 1996). It has also been shown that NO may participate in the regulation of the peristaltic activity of the tubules, a necessary process for sperm transport (Setchell et al, 1994). Furthermore, low levels of NO promote human sperm capacitation (Zini et al, 1995), whereas high levels decrease sperm motility and induce sperm toxicity (Rosselli et al, 1995). To our knowledge, no reports about effects of NO on Sertoli cell function are available. γGTP activity is a parameter of Sertoli cell function that may also be regarded as a marker of Sertoli cell differentiation (Hodgen and Sherins, 1973). Its activity increases with the age of the Sertoli cell donor (Lu and Steinberger, 1977) and is stimulated by FSH and by germ cell–derived factors of unknown nature (Schteingart et al, 1989). γGTP activity is involved in amino acid transport across the plasma membrane (Tate and Meister, 1981) and in the regulation of glutathione levels with subsequent protection from oxidative stress (Hanigan and Ricketts, 1993). Conclusive evidence showing that this enzyme activity is essential for normal sexual development has been obtained in γGTP-deficient mice (Lieberman et al, 1996). In a previous report, we have shown that γGTP activity can be regulated by cAMP-pkA-dependent, $Ca^{2+}$-calmodulin-dependent, and pkC-dependent pathways (Meroni et al, 1997). The present data suggest that γGTP activity can also be regulated by a NO-dependent pathway.

Cellular localization of the different NOS isoforms in the testis is a matter of debate. Burnett et al (1995) have proposed that in the rat testis, NADPH diaphorase activity and NOS immunoreactivity are confined to the vascular endothelium. Lissbrant et al (1997) have demonstrated the presence of eNOS and nNOS in the vascular endothelium and Leydig cells, respectively, but not in the Sertoli cells. On the other hand, Middendorff et al (1997) have shown that constitutive nNOS and eNOS and the inducible isoform iNOS are present in the human testis, particularly in Sertoli cells. The results presented herein demonstrate, by immunohistochemical techniques, the presence of the 2 constitutive isoforms of NOS in rat Sertoli cells cultured under basal conditions. Bauche et al (1998) have demonstrated that a mixture of interferon γ, TNFα, LPS, and

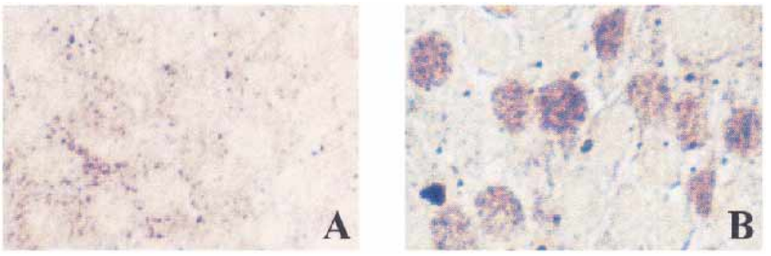


Figure 6. Immunohistochemical staining for p65 NF-κB. Immunohistochemical staining for p65 subunit of the transcription factor NF-κB was performed in basal and IL1β-stimulated cultures. In nonstimulated cells, p65 NF-κB immunoreactivity was only observed in the cytoplasm. In IL1β-stimulated cells, a strong p65 NF-κB immunoreactivity appeared in cell nuclei. The immunostaining results shown are representative of 3 independent experiments (450×).

IL1α induces iNOS mRNA expression in rat Sertoli cell cultures. However, no reports for the possible effect of IL1β on iNOS expression were available. We have observed that treatment with IL1β increases iNOS immunoreactivity. A similar stimulatory effect on iNOS for IL1β has been observed in cultured rat Leydig cells (Tatsumi et al, 1997).

An important signal of IL1β is the activation of the transcription factor NF-κB that is signaled via the type I IL1 receptor (Moynagh et al, 1993). NF-κB is a heterodimeric complex, usually consisting of p50 and p65 (Rel A) subunits, that functions as a pleiotropic regulator of many genes (Thanos and Maniatis, 1995). The p50/p65 heterodimer associates with IκB to form an inactive complex. Activation of NF-κB by LPS or cytokines requires the degradation of IκB (Begg et al, 1993). The free active heterodimer NF-κB translocates into the nucleus and activates gene expression. This process is required for cytokine induction of the human iNOS gene in liver, lung, and epithelial cell lines (Taylor et al, 1998). In the present study, we have demonstrated that IL1β induces NF-κB translocation, and we suggest that this process may be responsible for iNOS induction and NO production when Sertoli cells are stimulated with IL1β.

Delfino and Walker (1998) have determined that NF-κB is constitutively present and active in the nuclei of Sertoli cell cultures. Under basal conditions, we were unable to demonstrate by immunostaining the presence of NF-κB in the nucleus. Delfino and Walker (1998) have used a different isolation procedure and cultures were performed on Matrigel-coated dishes. These methodological differences can be accounted for by the discrepancy in the results obtained. Using an immunohistochemical technique, these authors found that nuclear localization of NF-κB proteins is regulated in a stage-specific manner. The highest level of NF-κB in the nucleus of Sertoli cells was found at spermatogenesis stages XIV–VII. We have isolated seminiferous tubule segments at different stages of the spermatogenic cycle by their transillumination pattern and found that the highest γGTP activity was present at stages II–VI (unpublished data). These observations suggest that both events might be functionally related.

Taken together, our results demonstrate that Sertoli cells are sites of NO production and activity. It is an attractive idea that NF-κB activation and its nuclear translocation as well as the expression of iNOS protein and subsequent NO production induced by IL1β are partly responsible for the increase in γGTP activity observed in Sertoli cell cultures. Assuming that this cytokine is produced by macrophages and Leydig cells in the interstitium, it is suggested that IL1β is involved in testicular cell-cell metabolic cooperation and that, among other possible signal transduction pathways, it utilizes a NO-dependent one to regulate Sertoli cell function.

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