Cyclooxygenase-2 in testes of infertile men: evidence for the induction of prostaglandin synthesis by interleukin-1β

As we previously reported, testes of men suffering from hypospermatogenesis and germ cell arrest or Sertoli cell-only syndrome show a major increase in the number of macrophages expressing interleukin-1β (IL-1β) and abundant expression of cyclooxygenase-2 (COX-2), the inducible isoform of the key enzyme in the biosynthesis of prostaglandins (PGs), in Leydig cells. In the present study we report [1] a positive correlation between IL-1β levels and COX-2 expression in testes of infertile patients, [2] the induction of COX-2 by IL-1β in mouse Leydig cells (TM3) and human macrophages (THP-1), and therefore [3] evidence for an IL-1β-dependent induction of testicular inflammatory states. (Fertil Steril® 2010;94:1933–6. ©2010 by American Society for Reproductive Medicine.)

Interleukin-1β (IL-1β) is a proinflammatory cytokine secreted by macrophages (MACs) (1, 2). In spite of its immune privileged status, the testis is not isolated from the immune system. Therefore, locally produced cytokines, as well as those in the general circulation, have the potential to exert effects at the testicular level (3). We have previously reported that human testicular MACs express IL-1β, and that the number of MACs is significantly increased in testicular biopsies of men with cases of idiopathic infertility revealing Sertoli cell-only (SCO) syndrome or severe hypospermatogenesis and germ cell arrest (GA) (2). It has been reported that IL-1β stimulates cyclooxygenase-2 (COX-2), the inducible isoform of the key enzyme in the biosynthesis of prostaglandins (PGs), in some tissues and cells such as granulosa–luteal cells and Leydig cell progenitors (4, 5). Because we reported that COX-2 is not detected in normal human testes but is expressed in testicular biopsies from infertile patients (6), in the present study we extended our previous works in this line of research and further investigated the IL-1β system in human testicular MACs and Leydig cells, its correlation with COX-2 expression, and the potential action of IL-1β on PG synthesis.

For the evaluation of human testicular biopsies from adult men (27–40 years old) with GA (n = 6) and SCO syndrome (n = 6), the Institutional Review Board (IRB) approval was obtained from the local Ethics Committees of Munich University (Germany), Durand Hospital (Buenos Aires, Argentina), and the Institute of Biology and Experimental Medicine, CONICET (Buenos Aires, Argentina). All participants granted written informed consent.

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The TM3 cells (American Tissue Culture Collection [ATCC], Riversville, MD), derived from immature mouse Leydig cells and THP-1 cells (ATCC) derived from an acute monocytic leukemia, were cultured in F12- Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical, St Louis, MO) supplemented with 5% horse serum and 2.5% fetal calf serum (FCS; Invitrogen Corporation, Carlsbad, CA) and RPMI1640 medium supplemented with 10% FCS and 0.05 mM 2-mercaptoethanol (Sigma Chemical), respectively. The THP-1 cells were differentiated into MACs with 30 nM of phorbol myristate acetate (Sigma Chemical) during 18 hours.

Polyclonal rabbit anti-COX-2 serum (Oxford Biomedical Research, Oxford, United Kingdom; 1:200), monoclonal mouse anti-IL-1β antibody (R&D Systems, Inc., Minneapolis, MN; 1:100), monoclonal mouse anti-human CD68 antibody (DAKO, Hamburg, Germany; 1:100), polyclonal rabbit anti-3β-hydroxysteroid dehydrogenase (3βHSD) serum (1:2,000), and the avidin-biotin-peroxidase system (Vector Lab, CA), were used for immunohisto/cytochemistry.

The laser capture microdissection and pressure catapulting (LMPC) technology developed by P.A.L.M. GmbH (Bernried, Germany) was used to microdissect CD68 and COX-2 immunoreactive cells from human testicular biopsies, as previously described (2, 6).

Total RNA was extracted from human testicular biopsies, cells isolated by LMPC and TM3/THP-1 cells, using the TRIzol reagent (Invitrogen), Purescript kit (Biozym, Hessisch Oldenburg, Germany), and QIAGEN RNeasy mini kit (QIAGEN Inc., Valencia, MO), respectively. The reverse transcriptase (RT) reaction was performed using dN6 random primers, followed by polymerase chain reaction (PCR) amplification (6). When required, a second PCR amplification using nested primers was also assayed. Semiquantitative PCR studies were performed using oligonucleotide primers for CD68 (first set: 5′-TGAACCCCAACAAAACC and 5′-GAG AGCAGGTCGAGGTG; heminested set: 5′-GTGCCCATCCC CACCTG and 5′-GAGAGCAGGTCGAGGTG), CD163 (first set: 5′-TGTAGCGGGAGAGTGGAA and 5′-CTGAGCAGGTCACT CCAG; nested set: 5′-TAATGGCTGGAGCATGGA and 5′-CAAAG AGCTGACTCATTC), tryptase (first set: 5′-TGCTGGAGCTGGA GGAGC and 5′-AGGTGCCATTCACCTTGC; nested set: 5′-GTG CTGGGTCACTGGCTG and 5′-CCCGCACACAGCATGTC), chymase (first set: 5′-AGGAGAAAGCCAGCCTGA and 5′-ATG CAGATTTTGTCTTCC; nested set: 5′-CTGGCTGTGGGGACA CTC and 5′-ATTGCCCACACACAGCTG), StAR (set: 5′-TGGAG AGGCTCTATGAAGAGC and 5′-GCCACGTAAGTTTGGTCT TAG), COX-2 (first set: 5′-TGTATGTATGAGTGTGGGA and 5′-GGCTTCCCAGCTTTTGTA; nested set: 5′-CTTACCCACTT CAAGGGA and 5′-GCCATAGTCAGCATTGTAAG), IL-1β (first set: 5′-CTGAAAGCTCTCCACCTC and 5′-CGCTTTTCCATCT TCTTCA; nested set: 5′-ACAAGTGGTATTCTCCATG and 5′-T CCACACTCTCCAGCTG), IL-1RI (first set: 5′-CTCCAGGATTC ATCAGCA and 5′-GACCCATTCCACTTCCAGTA; nested set: 5′-CTTGTGTGCCCTTATCTG and 5′-TGCTCTTCAGCCACAT TC), IL-1RII (first set: 5′-GGAATACAACATCACTAGGA and 5′-TTGTGACTGGATCAAAAATC; heminested set: 5′-CCTGTG ATCATTTCTCCC and 5′-TTGTGACTGGATCAAAAATC), IL-1Ra (first set: 5′-GCAAGATGCAAGCCTTCA and 5′-GTCTTCC TGGAAGTAGAA; nested set: 5′-TAACCAGAAGACCTTCTA and 5′-TGTGCAGAGGAACCATCC), and β-actin (set: 5′-GGAT GCAGAAGGAGATCA and 5′-CTAGAAGCATTTGCGGTG). Quantitative PCR reactions were conducted using SYBR Green PCR Master Mix, the ABI PRISM 7500 sequence detector System (Applied Biosystems, Foster City, CA), and oligonucleotide primers for COX-2 (5′- CTGGCGCTCAGCCATACAG and 5′- C ACTCATACATACACCTCGGT), IL-1β (5′-TCCCCAGCCCTTT TGTTGA and 5′-TAGAACCAAATGTGGCCGTG), and β-actin (5′- TCCCTGGAGAAGAGCTACGA and 5′-AGGAAGGAAGG CTGGAAGAG).

Immunoblottings were performed using a rabbit polyclonal anti-COX-2 antiserum (Cayman Chemical, Ann Arbor, MI; 1:250), a rabbit polyclonal anti-human IL-1β antiserum (Cell Signaling Technology Inc., Beverly, MA; 1:500), and a mouse monoclonal anti-actin antibody (Calbiochem, La Jolla, CA; 1:5,000) (7).

The PGD_2 and $PGF_{2\alpha}$ levels in the incubation media from TM3 and THP-1 cells, as well as the concentration of IL-1β in human testes, were assayed using commercially available kits (Cayman Chemical).

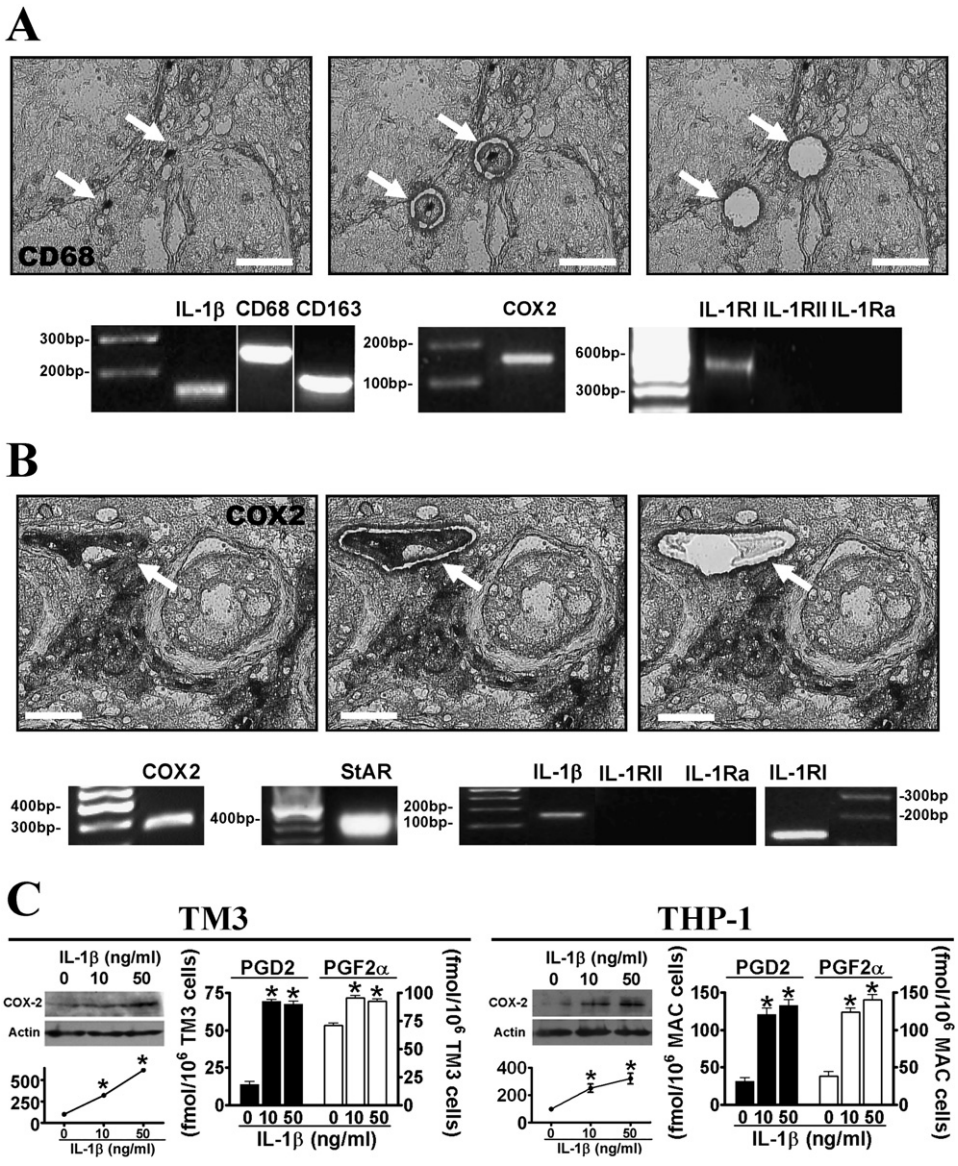
Cells immunoreactive for CD68, a classic MAC marker, were isolated from human testicular biopsies of infertile men by LMPC. Subsequent semiquantitative PCR analyses revealed the expression of IL-1β, its receptor IL-1RI, and COX-2 (Fig. 1A). In addition to MACs, we found another cell population from the interstitial compartment strongly stained with the antibody against COX-2 that shows characteristic features of Leydig cells. By LMPC and semiquantitative PCR studies, we demonstrated that these cells express IL-1β, IL-1RI, and StAR, a specific Leydig cell marker (Fig. 1B). Indeed, additional experiments showed immunohistochemical colocalization of COX-2 and 3β-HSD expression in all Leydig cells (data not shown). The lack of amplification of the antagonist receptor Ra and the scavenger receptor IL-1RII (Fig. 1A, B) might be due to methodological limitations of the LMPC/semiquantitative PCR techniques used in our studies, together with a low expression of those genes in the interstitial compartment of the male gonad (8).

Semiquantitative PCR and quantitative PCR assays showed a positive correlation between COX-2 messenger RNA (mRNA) expression and IL-1β mRNA expression in GA and SCO syndrome ($r = 0.87$ and 0.93 for semiquantitative PCR and quantitative PCR, respectively, $P<.05$). In addition, the expression of COX-2 protein by immunoblotting and the testicular IL-1β concentration in human testicular biopsies from infertile patients showed a correlation coefficient value of 0.69 ($P<.05$). We failed to identify IL-1β protein by immunoblotting, probably due to the low testicular IL-1β concentration (range between 100 and 1,000 fg/mg protein) or a low sensitivity of the antibody used.

Because of the obvious ethical limitations, we have no access to normal testes. Nevertheless, our previous studies performed in archival paraffin blocks of human testicular biopsies showing normal morphology, failed to detect expression of COX-2 by immunohistochemistry and in addition, a significantly lower amount of testicular MACs expressing IL-1β was detected compared with that found in infertile testes (2, 6). The lack of differences observed in either COX-2 or IL-1β expression among biopsies revealing GA and SCO syndrome (data not shown) could result from the similar MAC number detected in testes from both pathological groups (GA: 365.75 $\pm$ 21.5 MACs/mm^2 vs. SCO syndrome: 378.00 $\pm$ 97.5 MACs/mm^2) together with a potential stimulatory effect of IL-1β on interstitial COX-2 expression.

FIGURE 1

Using laser microdissection and pressure catapulting, CD68 immunoreactive macrophages (**A**) and cyclooxygenase-2 (COX-2) immunoreactive interstitial cells showing characteristic features of Leydig cells (**B**) were isolated from the testis of a patient with germ cell arrest and subjected to semiquantitative polymerase chain reaction (PCR) studies. Expression of COX-2 and the interleukin-1β (IL-1β) system in these cells has been detected. Bar, 40 μm. Comparable results were obtained when Sertoli cell-only biopsies were used (data not shown). (**C**) Mouse Leydig cells (TM3) and human macrophages (THP-1) were incubated for 6 hours in the presence or absence of IL-1β (10 or 50 ng/mL). The COX-2 protein expression was assayed by immunoblotting and results are expressed as arbitrary units relative to the control (basal conditions), which was assigned a value of 100, and normalized to actin. The prostaglandin (PG)D_2 and $PGF_{2\alpha}$ levels in the incubation media were determined by immunoassay. *$P<.05$ compared with the control group.



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Physiological studies cannot be performed on human testicular biopsies. To our knowledge, there are no human Leydig cell lines available and there are no accessible MACs cell lines derived from human testes. Therefore, non-human TM3 Leydig cells and non-testicular THP-1 MACs that express COX-2, IL-1β, IL-1RI, IL-1RII, and Ra (data not shown) were used to further characterize the interactions between the IL-1β system and COX-2/PGs. After 30 minutes, 3 hours (data not shown), and 6 hours (Fig. 1C) of incubation in the presence of IL-1β (10 and 50 ng/mL; Sigma Chemical), the expression of COX-2 protein and the production of PGD_2 and $PGF_{2\alpha}$ were significantly induced in TM3 and THP-1 cells (Fig. 1C). It is important to mention that reports from our group describe the modulation of testicular T production by PGD_2 and $PGF_{2\alpha}$ (9, 10). In addition, the actions of PGs and their receptors

have been described in Sertoli cells, germ cells, sperm, and fibroblasts of the wall of the seminiferous tubules (6, 11, 12).

Not only IL-1β, but also IL-1α, interacts with the IL-1RI receptor (3). This cytokine is produced by Sertoli cells (13), spermatocytes, spermatids (14), and testicular MACs (2). The expression of COX2 protein in TM3 and THP-1 cells showed a significant increase after 30 minutes of incubation in the presence of IL-1α (10 and 50 ng/mL; PeproTech, Rocky Hill, NJ), without apparent changes at longer incubation times (3 and 6 hours) (data not shown).

In conclusion, our current findings show the existence of an IL-1β system in MACs and Leydig cells of the human testis, and provide insights into how this system could participate in the induction of COX-2 expression and PG synthesis and therefore, in the development of local inflammation that might further deteriorate testicular function in patients with GA and SCO syndrome. Nevertheless, whether the testicular network formed by IL-1β, COX-2, and PGs has a biological relevance in the pathogenesis or maintenance of infertility states and therefore, a potential therapeutic value as targets in future strategies designed for the treatment of fertility disorders, remains to be further investigated.

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