

Effect of erythropoietin on Leydig cell is associated with the activation of Stat5 pathway

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Abstract

In order to elucidate whether erythropoietin (EPO) influences male reproduction, EPO activation via erythropoietin receptor (EPOR) of Stat5 was studied in Leydig cell cultures. RT-PCR analysis of mRNA that had been prepared from primary rat Leydig cell culture and the mouse Leydig tumor cell line (I-10 cells) each demonstrated amplification of the appropriate fragment for the respective genes. Both I-10 cells and primary rat Leydig cell cultures were treated with 1 U/ml recombinant human erythropoietin (rHuEPO) for various time periods upto 60 min, and cytoplasmic proteins, total cellular proteins, and nuclear extracts were isolated. Expression of Stat5b protein and phosphorylated Stat5b protein were investigated by Western blotting using anti-Stat5b pAb and anti-phospho-Stat5a/b pAb. Stat5b protein exists at the cytoplasmic level irrespective of exposure to rHuEPO, while phosphorylated Stat5b protein increases upon rHuEPO stimulation. DNA binding of nuclear protein to synthetic double-stranded oligonucleotides containing a DNA response element derived from PRL-inducible element (PIE) was examined using a gel mobility shift assay. The oligonucleotide representing the PIE of the rat β -casein promoter was demonstrated to bind to the nuclear extract of rHuEPO-treated cells, time dependently forming a single mobility shift complex X. These data suggest that EPO binds to a receptor expressed on the surface of Leydig cells. This EPOR regulates expression of specific gene, through a Jak–Stat pathway to influence male reproductive function.

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1. Introduction

Erythropoietin (EPO) is a serum glycoprotein hormone principally secreted by the kidney that is required for survival, proliferation, and differentiation of committed erythroid progenitor cells (Koury and Bondurant, 1988; Krantz, 1991; Jelkmann, 1992). EPO binds to a specific cell-surface receptor that is expressed on erythroid progenitors (Sawyer et al., 1987; Wognum et al., 1990). This erythropoietin receptor (EPOR) is functionally grouped with the cytokine receptors for other hematopoietic growth factors such as interleukins (ILs) and colony-stimulating factors, as well as GH. A family of protein tyrosine kinases known as Janus kinases (Jaks) that are associated with cytokine receptors play important roles in cytokine-dependent cell proliferation and

regulation of gene expression. EPO induces receptor dimerization, which leads to activation of Jak2. Activated Jak2, in turn, converts a latent cytoplasmic transcription factor, Stat5, to its active form by phosphorylation of a tyrosine residue. This activated Stat5 translocates into the nucleus, where it binds to specific DNA response elements in the promoter region of target genes and activates transcription (Darnell et al., 1994; Ihle, 1996).

Recently, EPO has been reported to act on some non-erythroid cells including endothelial cells (Anagnostou et al., 1990), smooth muscle (Gogusev et al., 1994), B lymphocytes (Kimata et al., 1991), rodent placental cells (Sawyer et al., 1989), embryonic stem cells (Schmitt et al., 1991), and megakaryocytes (Ishibashi et al., 1987). In vitro experiments using rat testis have demonstrated that EPO stimulates steroidogenesis in Leydig cells, increasing testosterone production via a direct, specific receptor-mediated mechanism (Mioni et al., 1992). Here we present, what we believe to be, the first reported evidence of testicular EPOR

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expression. We characterized EPO activation via EPOR of Stat5b in primary cultures of rat Leydig cells and the I-10 line of mouse Leydig tumor cells.

2. Material and methods

2.1. Materials

Immature male Sprague–Dawley (SD) rats (18-day-old) were purchased from Charles River Japan (Hino, Japan). Collagenase/dispase was obtained from Boehringer Mannheim (Mannheim, Germany). Recombinant human erythropoietin (rHuEPO) was purchased from Chugai Pharmaceutical (Tokyo, Japan). Anti-phospho-Stat5a/b polyclonal antibody (pAb) was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Stat5b(SC) pAb, raised against a peptide corresponding to amino acids 706–722 of mouse Stat5b, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HCG was kindly provided by the National Hormone and Pituitary Distribution Program of the US National Institute of Digestive and Kidney Disease (NIDDK); (Bethesda, MD).

2.2. Cell culture

All rats were killed by asphyxiation using CO₂. All studies were conducted in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Primary Leydig cells were prepared by methods described previously (Kanzaki et al., 1996). For the present studies, progenitor and immature Leydig cells were prepared from 20-day-old SD rats; 45% of the freshly isolated Leydig cells were immunoreactive for 3 α -HSD, and 42% were immunoreactive for 3 β -HSD (data not shown). These enzymes were used as indicators of the ratio of progenitor to immature Leydig cells, as well as the purity of the preparation and the maturational status of the cells (Peng et al., 1979; Klinefelter et al., 1987). Primary Sertoli cell was prepared from immature 18-day-old rats as described previously (Fujisawa et al., 1992).

Cells were cultured in serum-free medium supplemented with 2.5 μ g/ml bovine insulin (Life Technologies, New York, NY), 5 μ g/ml transferrin (Life Technologies), and 10 μ g/ml bacitracin (Sigma Chemical, St. Louis, MO). Leydig cells were cultured for 2 days as above, washed, and then stimulated with 1 U/ml rHuEPO for the times indicated in Section 3.

The I-10 cell line (Dainihon Pharmaceutica, Osaka, Japan) used in these experiments was maintained using a standard protocol. It is the luteinizing hormone—unresponsive Leydig tumor cell line derived from a spontaneous mouse tumor (Yasumura et al., 1966) and secrete steroid such as pregn-4-en-20 α -hydroxy-3-one (20 α -dihydroprogesterone) and pregn-4-en-3, 20-dione (progesterone), responding to

cAMP (Wolff and Cook, 1977). In addition, testosterone is not produced from I-10 cell lines.

For experiments, 10⁷ cells were plated in 100 mm polystyrene plates and grown in Ham's F-10 medium with 15% horse serum and 2.5% fetal bovine serum. After 3 days the medium was removed, cells were washed twice with Ca²⁺- and Mg²⁺-free PBS, and serum-free Ham's F-10 medium was added to cells. I-10 cells were incubated with or without rHuEPO.

All experiments were repeated three times using different primary cultures of Leydig and Sertoli cell preparations or different I-10 cell cultures with comparable results obtained in each replicate experiment. The results presented are typical for each experiment illustrated.

2.3. RNA isolation and RT-PCR assays

Total RNA was prepared from cultured cells with a commercial kit (Trizol, GibcoBRL, Paisley, UK) using a guanidine thiocyanate/phenol protocol with glycogen (20 μ g; Boehringer Mannheim) as a carrier.

For RT-PCR analysis, oligo(dT)-primed first-strand cDNA was synthesized from 2 μ g total RNA using an AMV reverse transcription kit (Takara, Kyoto, Japan) in a reaction volume of 20 μ l under conditions recommended by the manufacturer. PCR was performed with 2 μ l of the cDNA as a template in a final reaction volume of 100 μ l. The reaction was performed in 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 20 mmol/l Tris–HCl at pH 8.3, a 0.25 mmol/l concentration of deoxynucleotide triphosphates (dNTPs), a 0.75 μ mol/l concentration of each primer, and 0.05 U/ μ l Taq polymerase (Takara). The primer pair for mouse EPOR was 5'-GTTCAGTCCAAACAGGACA-3' (forward) and 5'-ATGAGAGACAGCGTCAAGATGAGA-3' (reverse), amplifying a 430-bp product; for rat EPOR, primers were 5'-GGGCTCCTATGACCACCCACA-3' (forward) and 5'-CTCACTGCCACTGGCTCCA-3' (reverse), amplifying a 550-bp product. We performed 30 cycles for both mouse and rat EPOR, each consisting of 30 s at 94 °C, 30 s at 60 °C, and 60 s at 72 °C using a thermal DNA cyclor.

2.4. Testosterone measurement

Testosterone concentrations in culture media were measured using a testosterone enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI).

2.5. Total cellular lysate isolation and immunoprecipitation

Cells (2 $\times$ 10⁷) were lysed in 1 ml of RIPA buffer (50 mM Tris–HCl at pH 7.5 containing 1% NP40, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin, and 1 mM sodium orthovanadate; Sigma) for 30 min on ice. Cellular debris was pelleted

by centrifugation at $12,000 \times g$ for 15 min. Cell lysates then were precleared for 1 h at 4°C with protein G–agarose beads (Santa Cruz) and were incubated overnight at 4°C with anti-Stat5b. Anti-Stat5b was captured by incubation for 1 h at 4°C with protein G–agarose beads and washed. Samples then were dissolved in $2\times$ Laemmli buffer and boiled for 2 min.

2.6. Cytoplasmic protein and nuclear extract preparation

Cells (10^7) were lysed in 1 ml of hypotonic buffer A (10 mM HEPES–KOH at pH 7.9, 10 mM KCl, 1.5 mM MgCl_2 , 0.5% NP-40, 2 mM dithiothreitol, 0.5 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin; Sigma). Cytoplasmic proteins were collected by centrifugation at $2000 \times g$ for 20 s. Pellets were dissolved and washed once with 1 ml of buffer A containing 20% glycerol. Nuclei were collected by centrifugation at $2000 \times g$ for 20 s. Then, nuclear proteins were extracted in 100 μl of high-salt buffer B (20 mM HEPES–KOH at pH 7.9, 0.55 mM KCl, 1.5 mM MgCl_2 , 2 mM dithiothreitol, and 20% glycerol, in addition to protease inhibitors and phosphatase inhibitors as for buffer A). This extraction was carried out for 30 min on ice with occasional vortex agitation. Supernatants (nuclear extracts) were collected by centrifugation at $12,000 \times g$ for 15 min.

2.7. Immunoblotting

Cytoplasmic proteins and immunoprecipitates were subjected to SDS–PAGE using 6.5% polyacrylamide gels under reducing conditions. Resulting bands were transferred electrophoretically to a nitrocellulose membrane (Millipore, Tokyo, Japan). Membranes were probed with antibodies diluted as follows: anti-Stat5b, 1:2500; and anti-phospho-Stat5a/b, 1:2000. Blots were developed with an enhanced chemiluminescence system for Western blotting (Amersham Pharmacia Biotech, Piscataway, NJ).

2.8. Electrophoretic gel mobility shift assay (EMSA)

The DNA probe used for the gel mobility shift assay represented the PRL-inducible element (PIE; 5'-AGATTCTAGGAATTCAATCC-3'; Santa Cruz, sc-2565). The PIE probe was end-labeled on one strand using T4 polynucleotide kinase (Takara) and [γ - ^{32}P] (Amersham Pharmacia Biotech). Nuclear extracts were incubated at room temperature with labeled oligonucleotide probe in binding buffer with poly(dI–dC) acid (Amersham Pharmacia Biotech) for 30 min, before separation by electrophoresis through a 4.5% polyacrylamide gel in $0.5\times$ Tris–borate–EDTA. Signals were visualized with an enhanced chemiluminescence system.

3. Results

3.1. Expression of mRNA for the EPOR genes

The presence of mRNA products of the EPOR genes was assessed in both primary cultures of rat Leydig cells and the mouse Leydig tumor cell line (I-10 cells), using primers specific for rat EPOR and mouse EPOR. RT-PCR analysis of mRNA that had been prepared from primary rat Leydig cell culture and I-10 cells each demonstrated amplification of the appropriate fragment for the respective genes (Fig. 1).

3.2. Testosterone production

We measured testosterone in culture media after stimulating primary rat Leydig cells with 100 ng/ml hCG and 1 U/ml rHuEPO. Both hCG (NIDDK, Bethesda, MD) and rHuEPO induced a significant increase in culture media testosterone concentration that generally was time dependent (Fig. 2).

3.3. Tyrosine phosphorylation of Stat5 proteins in I-10 cells and primary rat Leydig cells

Both I-10 cells and primary rat Leydig cell cultures were treated with 1 U/ml rHuEPO for various time periods upto 60 min, and cytoplasmic proteins were isolated. Expression of Stat5b protein was investigated by Western blotting. Stat5b immunoreactive bands were expressed at all times, and their intensity did not change (Fig. 3).

We, next, studied tyrosine phosphorylation of Stat5b proteins in I-10 cells and primary rat Leydig cell cultures treated with EPO. Cells were exposed to the hormone as above. Total cellular proteins were immunoprecipitated with anti-Stat5b pAb, and tyrosine-phosphorylated Stat5b was detected by Western blotting using anti-phospho-Stat5a/b pAb. Tyrosine phosphorylation of the Stat5b band was seen in both EPO-treated cell preparations by 5 min. The amount of phosphorylated Stat5b decreased after 30 min, but phosphorylation of the band still was apparent at 60 min (Fig. 4).

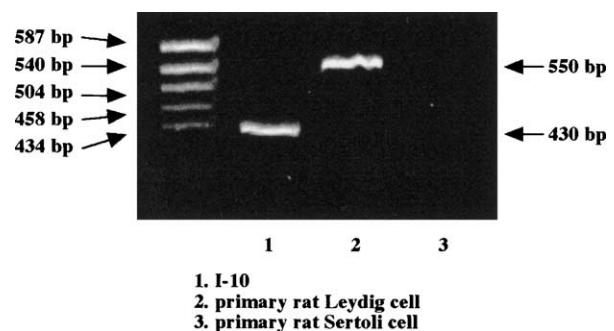


Fig. 1. Expression of mRNA for the erythropoietin receptor in mouse-derived I-10 cells, primary rat Leydig cell cultures, and primary rat Sertoli cell cultures. Total RNA was isolated, and 2 μg was used for RT-PCR with primers for rat or mouse EPOR. PCR-amplified products were separated by electrophoresis through acrylamide gels and were visualized by ethidium bromide staining.

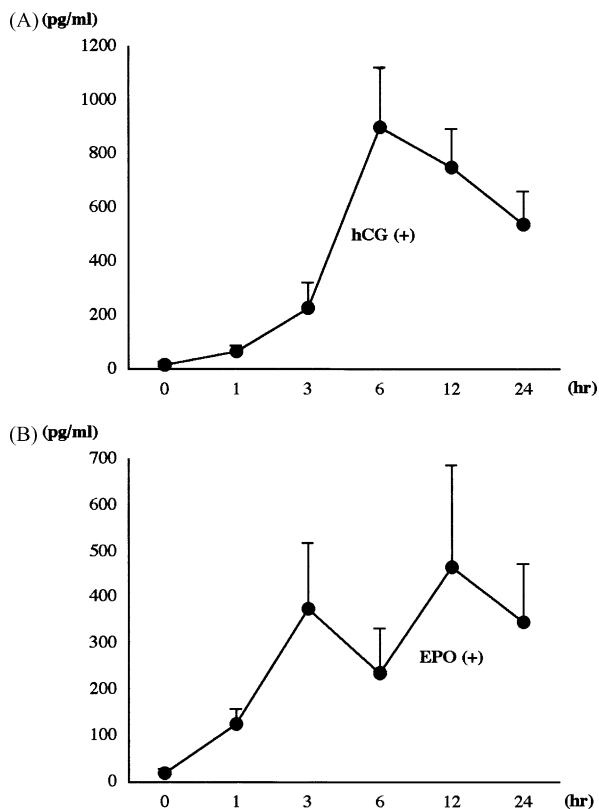


Fig. 2. Testosterone production by primary cultures of rat Leydig cell treated with hCG or erythropoietin. In A, primary cultures of rat Leydig cells were incubated with 100 ng/ml hCG for the times indicated. Testosterone concentrations in culture media were measured by EIA. In B, primary rat Leydig cell cultures were incubated with 1 U/ml rHuEPO for the times indicated. Testosterone similarly was measured in culture media by EIA.

3.4. Binding of Stat5 proteins to a gamma-activated site in EPO-treated I-10 cells and primary rat Leydig cell cultures

We, next, studied whether the effects of EPO on Stat5b protein tyrosine phosphorylation were associated with enhanced binding of Stat5b to gamma-activated site (GAS) elements. DNA binding of nuclear protein from I-10 cells and primary rat Leydig cell cultures to synthetic double-stranded

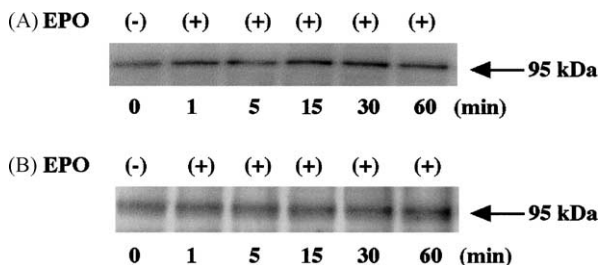


Fig. 3. Stat5b protein expression in primary rat Leydig cells and I-10 cells. In A, primary rat Leydig cells cultures were incubated with erythropoietin (1 U/ml rHuEPO) for the times indicated. Cytoplasmic proteins were immunoblotted with anti-Stat5b (dilution, 1:2500). In B, I-10 cells were studied by same protocol as for A.

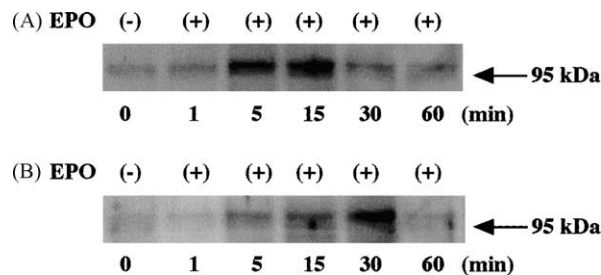


Fig. 4. Erythropoietin-promoted tyrosine phosphorylation of Stat5b in cultures of primary rat Leydig cells and I-10 cells. In A, rat Leydig cells were incubated with 1 U/ml rHuEPO for the times indicated. Whole-cell lysates were immunoprecipitated with anti-Stat5b (pAb). Immunoprecipitated proteins were immunoblotted by anti-phospho-Stat5a/b (dilution, 1:2000). In B, I-10 cells were studied as for A.

oligonucleotides containing a DNA response element derived from PIE was examined using a gel mobility shift assay. The oligonucleotide representing the PIE of the rat β -casein promoter binds predominantly to nuclear extracts of EPO-treated cells and forms a single mobility shift complex X. The intensity of complex X increased in a time-dependent manner in both I-10 cells and primary rat Leydig cell cultures (Fig. 5).

4. Discussion

Several authors have suggested that EPO may directly influence Leydig cell testosterone production through a specific receptor-mediated mechanism, but EPOR had not been

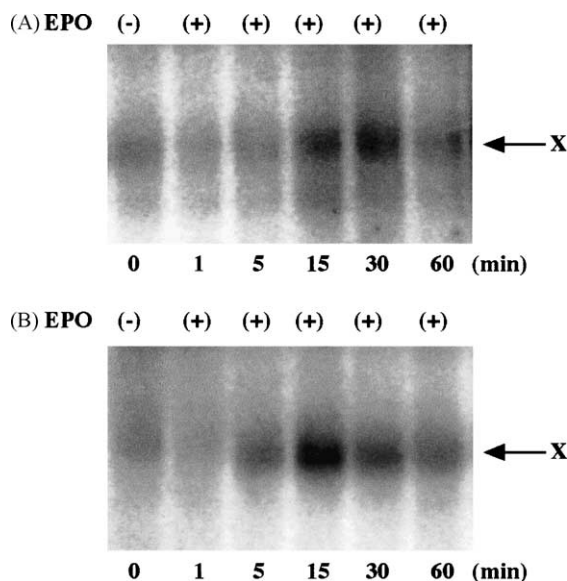


Fig. 5. Erythropoietin-modulated Stat5 protein binding to the PIE oligonucleotide in primary-culture rat Leydig cells and in I-10 cells. Nuclear extracts were subjected to EMSA with the PIE probe. In A, rat Leydig cells were incubated with 1 U/ml rHuEPO for the times indicated. Nuclear extract and PIE probe formed a specific protein-DNA complex X. In B, I-10 cells were studied as in A.

demonstrated in Leydig cells or other testicular cells. In the present study, we demonstrated the presence of EPOR in primary cultures of immature rat Leydig cells and in the I-10 mouse Leydig tumor cell line. We also demonstrated that like hCG rHuEPO stimulates testosterone production in vitro. This result is consistent with previously reported findings. Therefore, the EPOR protein in Leydig cells is a functioning receptor. Several clinical reports indicate that long-term therapy with rHuEPO improves sexual function in uremic men undergoing hemodialysis (Evans et al., 1990; Bommer et al., 1989). Moreover, rHuEPO therapy has been found to induce an increase in plasma testosterone, reduction of plasma FSH and LH, normalization of plasma PRL, and restoration of FSH responses to exogenous administration of GnRH in men with chronic renal failure (Schaefer et al., 1988; Kokot et al., 1995). On the other hand, several studies did not confirm normalization of PRL, or increases in testosterone in uremic patients treated with rHuEPO (Watsinger et al., 1991; Steffensen and Aunsholt, 1993). The influence of rHuEPO on hormones affecting sexual function in this population thus remains in dispute. EPO initiates a hematopoietic response by binding to its EPO receptor expressed on the surface of immature erythroblasts (Sawyer et al., 1987; Wognum et al., 1990). After ligand binding, the EPOR activates a cytoplasmic protein tyrosine kinase, Jak2, which in turn activates a transcription factor, Stat5. This signal transduction cascade induces development of early erythroid progenitors into mature erythroblast cells (Penta and Sawyer, 1995). Erythroid progenitors die in the absence of EPO; their genomic DNA is degraded into oligonucleosomal fragments indicating apoptosis (Koury and Bondurant, 1990).

Members of the signal transducers and activators of transcription (Stat) family are latent transcription factors involved in cytokine, hormone, and growth-factor signaling. Stat5 originally was cloned as a transcription factor for milk protein genes in the mammary gland. In the search for the identification of the PRL response element, GAS sequence (TTCNNGGAA) in the promoter of the β -casein gene was identified, as well as a putative transcription factor termed mammary gland factor (MGF) (Schmitt-Ney et al., 1991). MGF was cloned from sheep mammary tissue and identified as Stat5, a new member of the Stat protein family (Wakao et al., 1994). In the mouse, Stat5 exists as two isoforms (5a and 5b) showing 96% similarity at the amino acid level; these proteins differ mainly at the carboxyl terminus, resulting in differential transcriptional activation (Liu et al., 1995). In many tissues expressing Stat5, this protein can be activated by GH and PRL as well as several cytokines including EPO (Toukine et al., 1995; Ram et al., 1996). A previous study showed evidence of testicular Stat5 protein expression and characterized GH and PRL activation of Stat5b in primary cultures of testicular Leydig cells, in the testis (Kanzaki and Morris, 1998).

In the present experiments, we identified and characterized Stat5 protein in primary cultures of immature rat Leydig cells as well as I-10 mouse Leydig tumor cells. We found

that in both Leydig cell preparations, Stat5 protein exists at the cytoplasmic level irrespective of exposure to rHuEPO, while phosphorylated Stat5 protein increases upon rHuEPO stimulation. In an EMSA, the oligonucleotide representing the PIE of the rat β -casein promoter was demonstrated to bind to the nuclear extract of rHuEPO-treated cells, time dependently forming a single mobility shift complex X. These results indicate that rHuEPO mediates tyrosine phosphorylation of the Stat5 protein and DNA binding to GAS elements in rat Leydig cells and in the I-10 cell line.

Taken together, our data suggest that EPO binds to a receptor expressed on the surface of Leydig cells. This EPOR regulates expression of specific gene, through a Jak–Stat pathway to influence male reproductive function.

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