Evidence for specific binding and stimulatory effects of recombinant human erythropoietin on isolated adult rat Leydig cells

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The presence of specific binding of recombinant human erythropoietin and its effect on testosterone production were evaluated in isolated intact adult rat Leydig cells. Maximal specific binding was observed after 135 min incubation at 34°C. Scatchard analysis of the binding data revealed two distinct classes of binding sites for [125I]-recombinant human erythropoietin with dissociation constant of (Kd1) 1.9 x 10^-10 mol/l and (Kd2) 1.37 x 10^-8 mol/l respectively and binding capacity of (Bmax1) 12.3 fmol/10^6 cells and (Bmax2) 42.8 fmol/10^6 cells, respectively. GnRH, hCG, IGF-I and EGF did not induce any modification of recombinant human erythropoietin-specific binding. Recombinant human erythropoietin added to isolated adult rat Leydig cells exerted a stimulatory effect on testosterone production reaching its maximal effect at the dose of 10^-10 mol/l (testosterone production from 14.9 + 1.7 to 45.1 ± 6.2 pmol/10^6 cells/3 h). Addition of anti-recombinant human erythropoietin serum completely blocked the recombinant human erythropoietin-stimulated testosterone production. These results show that purified adult rat Leydig cells possess recombinant human erythropoietin specific binding, and suggest that this glycoprotein directly influences rat Leydig steroidogenesis.

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Growth factors, a family of polypeptides with molecular weights of about 40 kD or less, are intimately involved in the regulation of growth, differentiation and development of mammalian cells. Most of these peptides exert their effects, by paracrine or endocrine mechanisms, activating specific cell surface receptors (1-5). Recent evidence suggests that several growth factors are involved in the regulation of testicular functions. In particular, it has been observed that, e.g., epidermal growth factors (EGF), insulin-like growth factor-I (IGF-I), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF) and insulin were able to influence Leydig steroidogenesis through the binding to specific plasma membrane receptors (6-10). Erythropoietin (EPO), a 34 kD glycoprotein hormone, principally secreted by the kidney, is an essential growth factor for normal erythropoiesis (11-15). It regulates the proliferation and terminal differentiation of pro-erythroblasts and their immediate precursors, the colony-forming unit erythroid cells (CFU-e), acting on specific receptors (15-17). Recently, it has been observed that the receptors of erythropoietin, EGF, IGF-I and interleukins 2, 3, 4 and 6 are homologous in their structural features, in accordance with the existence of a superfamily of growth factors (18, 19). To date, there are no clear physiological explanations for this receptor-structure homology, unless these factors may influence cellular functions activating a common signal transduction pathway. The aim of this study was to investigate whether erythropoietin, as well as other growth factors (6-10), influences steroidogenesis in isolated adult rat Leydig cells and if this effect is mediated by specific receptors.

Materials and methods

Materials

Medium-199 with Hanks’ salts with L-glutamine, penicillin and streptomycin were obtained from the Grand Island Biological Co (Grand Island, NY); collagenase (type II), BSA (fraction V), HEPES, soybean trypsin inhibitor (type 1s), TRIS (hydroxymethyl)-aminoethan, and GnRH (Gly-6) were from Sigma (St Louis, MO); Percoll was from Pharmacia Fine Chemicals AB (Uppsala, Sweden); highly purified hCG (48600 UI/g) was from Serono (Rome, Italy); EGF (0.1 g/l) was from BioMacor (Rehovot, Israel); human recombinant IGF-I (0.1 g/l) was from Calbiochem (San Diego, CA). rHuEPO (10^6 U/l) was from Cilag GmbH (Aisbach-Hahnlein, Germany); human erythropoietin was produced by recombinant DNA technology and obtained by inoculation of isolated gene in mammalian cell cultures. The specific erythropoietin activity was approximately 110,000 U per mg of glycoprotein and determined by both in vitro and in vivo 59Fe-uptake methods (20). High specific activity [125I]-labeled erythropoietin was
obtained from Amersham, UK (specific activity 3000–4000 Ci/mmol: 111–148 TBq/mmol). Rabbit anti-rHuEPO serum, which neutralized about 1.5 U of rHuEPO at a dilution of 1:200, was generously provided by Dr V Ferrari (Department of Pediatrics, University of Padua, Padua, Italy).

**Isolation and purification of Leydig cells**

Adult male rats of the Sprague-Dawley strain (280–320 g) were used. Testicular interstitial cells were prepared through decapsulation and collagenase digestion. Briefly, 12–14 decapsulated testes were placed in sterile polyethylene vials (50 cm³) containing medium-199 (3 ml/testis) with Hanks’ salts and L-glutamine, 0.2% BSA (fraction V), 1 g/l collagenase (type II), and shaken (90 cycles/min), in controlled atmosphere (pO₂ 95%–pCO₂ 5%) at 34°C. After 15–20 min, 15 ml of medium-199 was added to each vial and the suspension was filtered through sterile nylon gauze (mesh 0.5–0.8 mm) into a sterile 50 cm³ centrifuge tube. Erythrocytes (about 75–85%) were removed by the addition of a Percoll cushion (5 ml of 60% v/v, in the bottom of each vial), followed by centrifugation for 10 min at 800 × g at 22°C. Cells, at the medium-199/Percoll 60% interface, were then carefully aspirated, rinsed twice in 5 ml of medium-199. After centrifugation at 100 × g for 10 min at 22°C, cells were resuspended in medium-199 to give approximately 40 × 10⁹ cells/l. Purification of Leydig cells was obtained by layering 5 ml of crude interstitial cell suspension (20–25 × 10⁹ cells/l) on top of each vial, containing a previously prepared discontinuous Percoll density gradient (20–60%), and then centrifuged at 800 × g for 20 min at room temperature. The fractions were collected from the bottom of the tubes with a peristaltic pump (minipuls-3 Gilson, Villiers, France), and then rinsed twice in isotonic medium-199 to remove any residual Percoll. Purified Leydig cells (85–92% staining positively for 3-β-OH-steroid-dehydrogenase activity (21)) were resuspended in medium-199. Cell concentration (about 1.0–1.2 × 10⁶ Leydig cells/testis) and viability (over 90%) were determined using a hemocytometer and trypsin blue method, respectively.

**Measurement of radiiodinated erythropoietin binding to intact rat Leydig cell**

Binding assays were performed with [¹²⁵I]-rHuEPO in purified adult rat Leydig cell suspensions. Aliquots (0.25 ml) of Leydig cell suspension (1.0 × 10⁹ cells/l) were incubated with 2.0 nmol/l [¹²⁵I]-rHuEPO, with or without unlabeled rHuEPO, for various periods of time from 0 to 180 min and at different temperatures (4°C, 34°C). Both cells and rHuEPO were in binding buffer consisting of medium-199 with 25 mmol/l HEPES (pH 7.4) and 1% BSA (fraction V). For equilibrium studies, aliquots of purified intact adult Leydig cells (final concentration 1.0 × 10⁹ cells/l) were incubated, at 34°C for 3 h, in sterile multi-well tissue culture dishes, with medium-199 added to a final volume of 0.25 ml. Each well containing an increasing concentration of [¹²⁵I]-rHuEPO (2.7 pmol/l to 3.8 nmol/l giving 28,000–1,800,000 cpm). Non-specific binding was measured in the presence of a 1000-fold molar excess of unlabeled rHuEPO. After incubation, cell suspensions were transferred to microcentrifuge tubes and washed twice with 2 ml of ice-cold Dulbecco’s phosphate buffered saline with 1% BSA (PBS-1% BSA pH 8.0). After centrifugation at 800 × g for 5 min at 4°C, radioactivity was measured, both in the supernatant and in the pellet in a gammaspectrometer for 120 sec. With the same experimental conditions, we determined the intracellular radioactivity in the cells both in binding and equilibrium studies: after incubation with [¹²⁵I]-rHuEPO in the presence and absence of 1000-fold molar excess of unlabeled rHuEPO, each aliquot was rinsed twice in PBS-1% BSA (pH 7.4). Then, 2 ml of 50 mmol/l glycine-buffered saline (pH 3) were added for 2 min into all the tubes on ice to remove surface-bound rHuEPO. After washing twice in PBS-1% BSA, the cells were resuspended with 0.5 ml of medium-199 and centrifuged at 100 × g for 15 min at 4°C. Radioactivity was measured both in the supernatant and in the pellet, previously dissolved in 0.5 mmol/l NaOH. The bound radioactivity in microcentrifuge tube without cells was less than 0.3% of the total counts added. Displacement studies were performed with rHuEPO and unrelated peptides (hCG, GnRH, EGF, IGF-I) at doses ranging from 10⁻¹⁰ mol/l to 10⁻⁷ mol/l.

**Effects of rHuEPO on testosterone production**

Aliquots (0.5 ml) of purified Leydig cell suspensions, prepared as described above (1.0 × 10⁹ cells/l), were incubated in medium-199 with Hanks’ salts, L-glutamine, TRIS, 0.1% BSA (fraction V), penicillin (10⁴ U/l), streptomycin (1 g/l), at pH 7.4. in polyethylene sterile tubes containing rHuEPO, dissolved in medium-199, at doses ranging from 10⁻¹¹ to 10⁻⁹ mol/l, in a shaking-rack (90 cycles/min) at 34°C for 3 h, in a controlled atmosphere (pO₂ 95%–pCO₂ 5%). To a different set of tubes, under the same experimental conditions, anti-rHuEPO serum was added. Furthermore, the effects of hCG on rHuEPO-stimulated testosterone production were evaluated, incubating two doses of hCG, 10³ ng/l and 10⁴ ng/l respectively, in different aliquots of Leydig cell suspension containing rHuEPO at the doses ranging from 2 × 10⁻¹² to 10⁻¹⁰ mol/l, at the time and conditions reported above. After incubation, all the tubes were immersed in an ice-water bath (0°C) and then centrifuged at 1500 × g for 15 min at 4°C. Each supernatant was immediately stored at −20°C until hormone assay. Cell viability (over 90%) in the resuspended pellets from each tube was determined by the trypsin blue method.
Results

Min. incubation dependent rHuEPO amount the

The EBDA experiments, for maximal and

Statistical analysis

For the analysis of binding and equilibrium data, each point was considered as the mean of three independent experiments, each done in duplicate. Scatchard analysis and displacement studies were performed using the EBDA and Ligand computer programs (23-24). For hormone production, results of three independent experiments were considered and expressed as mean±SEM and analysed by Student’s t-test for unpaired data.

Hormone measurements

Testosterone production of Leydig cell incubations was measured by the RIA method as previously described (22). The sensitivity was estimated as 3.6 fmol/l and intra- and interassay coefficients of variation were 7.8% and 7.0% respectively.

Furthermore, the intracellular radioactivity, in the Leydig cells, was calculated at both 34°C and 4°C. After glycerol treatment the extracellular radioactivity was completely removed and no activity was found in the supernatant of resuspended cells (data not shown).

The saturation curves of [125I]-rHuEPO binding to rat Leydig cells and of its intracellular measurement are shown in Fig. 2. The incubation of Leydig cell suspensions with increasing concentration of [125I]-rHuEPO demonstrated the existence of a saturable binding. Furthermore, Scatchard analysis of these binding data revealed the existence of two classes of binding sites: (a) one with high affinity (Kd1 = 1.9 x 10^-10 mol/l) and binding capacity (Bmax1) of 12.3 fmol/10^6 cells; (b) the other with low affinity (Kd2 = 1.37 x 10^-8 mol/l) and binding capacity (Bmax2) of 42.8 fmol/10^6 cells respectively (Fig. 3). Displacement study of [125I]-rHuEPO, obtained with incubation of unrelated peptides (hCG, GnRH, EGF and IGF-I), did not evidence any effect on the specific rHuEPO binding to intact rat Leydig cells (Fig. 4).

Hormonal production

rHuEPO influenced testicular steroidogenesis, inducing a stimulatory effect on testosterone production in isolated adult rat Leydig cells. Fig. 5 shows the effect of increasing doses of rHuEPO on Leydig cell steroidogenesis. This glycoprotein begins to stimulate testosterone production at the dose of 10^-11 mol/l and produces its maximal effect at 10^-10 mol/l, maintaining a steady state up to 10^-9 mol/l. When rHuEPO was incubated...
Fig. 2. Intact rat Leydig cells were incubated with increasing concentrations of [¹²⁵I]-rHuEPO (●). The intracellular radioactivity was also determined (▲). These curves are obtained with the mean of three different experiments done in duplicate. Non-specific binding for each concentration was determined by the addition of 1000-fold excess of unlabeled hormone.

Fig. 3. Scatchard analysis of the specific binding of [¹²⁵I]-rHuEPO to purified adult rat Leydig cells is shown. Two specific binding sites are observed: Kd₁ = 1.9 × 10⁻¹⁰ mol/l, with about 1800 sites/cell, and Kd₂ = 1.37 × 10⁻⁸ mol/l, with about 6400 sites/cell. The points summarized three independent experiments, each done in duplicate. Increased amounts of radiolodinated rHuEPO were added to the cells with or without an excess of unlabeled rHuEPO for each point.

with its neutralizing antiserum, rHuEPO-stimulated testosterone production was completely blocked. The stimulatory effect of the different doses of rHuEPO on Leydig cell steroidogenesis was significantly enhanced when hCG was added in the medium at low (10³ ng/l) and higher (10⁴ ng/l) concentrations (Fig. 6).

Discussion
Our data show that rHuEPO specifically binds to intact rat Leydig cells and exerts a stimulatory effect on testosterone production. The amount of this binding is influenced by the temperature, reaching the highest values at 34°C, as well as for hCG and other hormones.
Fig. 4. Displacement study was performed in Leydig cells, incubating at 34°C for 3 h [\textsuperscript{125}I]-rHuEPO, at the dose of 1.0 nmol/l, with increasing concentrations of unlabeled rHuEPO (●), GnrH (▲), hCG (△), IGF-I (□) and EGF (*) respectively. Each point represents the mean of three experiments.

Fig. 5. The effects of rHuEPO on testosterone production in purified adult rat Leydig cells is summarized. Increasing concentrations of rHuEPO were incubated at 34°C for 3 h. In the same experimental conditions, through the addition of anti-rHuEPO serum (Ab-rHuEPO dashed line; for dilution see Materials and Methods), the stimulatory effect of rHuEPO on testosterone production was completely blocked. Statistical differences were: *p<0.05 versus control; **p<0.01 versus control.

At high temperature, rHuEPO may be internalized, resulting in an overestimation of cell surface binding; however, the low measurement of the internalized [\textsuperscript{125}I]-rHuEPO (rate of internalization < to 0.05 fmol/10\textsuperscript{6} cells/min at 34°C) excludes this possibility.

The Scatchard analysis of [\textsuperscript{125}I]-rHuEPO binding to intact rat Leydig cells revealed two classes of binding sites, with respectively: (a) high affinity (K\textsubscript{d1} = 190 pmol/l) and binding capacity of 12.3 fmol/10\textsuperscript{6} cells and (b) low affinity (K\textsubscript{d2} = 13.7 nmol/l) and binding capacity of 42.8 fmol/10\textsuperscript{6} cells. These binding data can be compared with those obtained by several authors in other cellular systems (28–31). In particular, it has been observed that erythropoietin exerts its effects on spleen
and liver erythroid progenitor cells, through the activation of specific binding of comparable affinities (Kd < nmol range) to those observed in our study, suggesting an involvement of similar receptors (32–34). Recent observations have shown that some growth factors, rHuEPO included, possess a common binding domain (18, 19, 35); therefore, a cross-linking reaction of rHuEPO with these receptors may exist (36–39). However, our displacement study with several unrelated peptides, such as hCG, GnRH, EGF and IGF-I, suggests the specificity of rHuEPO binding to rat Leydig cells. In accordance with this observation, rHuEPO did not induce any stimulatory effect on rat Leydig cells in the presence of rHuEPO-antiserum. Our present data show that rHuEPO was able to stimulate rat Leydig cell testosterone production. The stimulatory activity of this glycoprotein is dose-related up to $10^{-11}$ mol/l, reaching a steady state at higher doses. The most effective rHuEPO concentrations were comparable to those observed in rat plasma levels during pharmacokinetic studies of a single iv bolus of 30–60 U/kg of rHuEPO (40, 41). The maximal stimulatory effect, observed at low doses (under $10^{-11}$ mol/l), may be due to the activation of a partial fraction of the total specific receptor sites. In fact, in adult rat Leydig cells, this phenomenon is well known with other physiologic agonists, as well as LH or hCG, that are able to reach the maximal stimulatory effect binding only 1% of their specific receptors (21, 42).

In the presence of hCG, rHuEPO still exerts a stimulatory effect on testosterone production, suggesting that this hormone influences rat Leydig cell steroidogenesis without interfering with hCG-activated receptor systems and involving different post-receptor mechanisms. A direct stimulatory effect of testosterone on colony forming units–erythroid cells has been demonstrated and several clinical studies have shown that androgens have been commonly used in the treatment of various hemopoietic disorders (43, 44). Our results suggest that erythropoietin may influence hemopoiesis, also involving the most important endogenous source of androgen production in the male, the testis. This hypothesis is further supported by recent data showing that in male patients undergoing chronic hemodialysis the treatment with recombinant human erythropoietin improved sexual function and increased basal plasma testosterone levels (45, 46). These observations have justified emphasizing the normalization of the high plasma PRL levels or with the improvement of anemia (47, 48). However, in view of our data, a direct rHuEPO effect on testicular functions cannot be ruled out.

In conclusion, our study shows that rHuEPO influences rat Leydig steroidogenesis by stimulating testosterone production through a direct specific receptor mechanism and suggests a possible linkage between the erythropoietic system and testicular function.

References

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