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The Relaxin-Like Factor Is a Hormone

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The relaxin-like factor (RLF) circulates in the bloodstream of humans, interacts with a membrane protein with all the characteristics of ligand-receptor binding, and must therefore be considered a hormone by definition. The polyclonal antibody raised against synthetic human RLF showed no crossreactivity to other structurally related hormones, like insulin and relaxin. The sensitivity of this assay (ED_{50} at 100 p*M*) allowed the direct measurement of RLF concentrations in serum. The highest levels were detected in the serum of postpuberty males (190 p*M*), whereas in females and children, the RLF concentration was one order of magnitude lower.

Key Words: Relaxin-like factor, RLF; Leydig insulin-like peptide; radioimmunoassay; puberty.

Introduction

Testicular Leydig cells express a relaxin-like factor (RLF), previously named Leydig insulin-like peptide (LEY I-L) *(1,2)*. Although predominantly expressed in testis, the RLF message was also found in the human corpus luteum and trophoblast *(3)*. More recently, low but distinct expression of RLF messenger RNA was detected in the ovaries of mice *(4,5)*, but high levels were reported in bovine and ovine ovaries during cycling and pregnancy *(6,7)*. The localization of hormone receptors in female tissues, such as uterus, suggested regional overlaps of effects with relaxin. In addition, using chemically synthesized RLF, we also found the brain to be a target of RLF *(8)*.

Ivell et al. *(9)* and Adham et al. *(10)* suggest that the RLF may be an autocrine factor with its major function in the Leydig cells, but the presented studies suggest that it is a circulating hormone in both genders with preponderance in postpuberty males.

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Results and Disscussion

Circulating RLF concentrations in human blood were determined by RIA. Polyclonal antihuman RLF antibody was raised in rabbits against synthetic human RLF and characterized by using ^{125}I-RLF as tracer. The high affinity of our antibody against human RLF (ED_{50} of 100 p*M*, 630 ng/L), extended the lower level of detectability to 10 p*M*. The specificity of the antihuman RLF antibody was confirmed by measuring crossreactivity to hormones of similar structure, i. e., insulin, human relaxin, and mouse RLF. As depicted in Fig. 1, neither insulin nor human relaxin displaced antibody-bound ^{125}I-RLF, and the synthetic mouse RLF showed a surprisingly weak reaction. Clearly, the rabbit anti-hRLF antibody has the requisite specificity for the detection of RLF levels in human serum.

The RLF levels depicted in Fig. 2 show significant differences between pre- and post-puberty males, whereas postpuberty females retain the same low levels as observed in all children. In spite of large variations within the adult male population RLF values do not overlap with those of the female population (Fig. 3).

Using 100 µL of serum in postpuberty males, tracer binding was suppressed by 40–70% of the total binding, but in post puberty females and children, binding was suppressed by 15–30%. The presence of higher RLF levels in post puberty males is in harmony with the proposal that RLF has sperm maturation activity *(10)* as one of its functions.

Addition of excess hRLF to the serum displaced all ^{125}I-hRLF specifically bound to the antibody, whereas addition of human relaxin had no effect (Fig. 4). This experiment excludes artifacts owing to crossreactivity or nonspecific binding proteins from consideration. Serial dilutions of synthetic hRLF in low-activity female serum resulted in a dose–response curve that is indistinguishable from one run in buffer only. These data confirm that the antibody is specific for RLF and that in human serum, hRLF is fully accessible to the antibody.

The RLF appears to be a specific marker for Leydig cells *(5)* but so far specific RLF receptors have only been found in those female tissues that also contain relaxin receptors *(8)*. The work of Zimmermann et al. *(5)* suggests a male-specific autocrine or paracrine role for RLF, whereas the presence of specific receptors in females speaks for a more general activity of the new factor. In order to initiate the search for the functional potential of RLF it seemed impor-

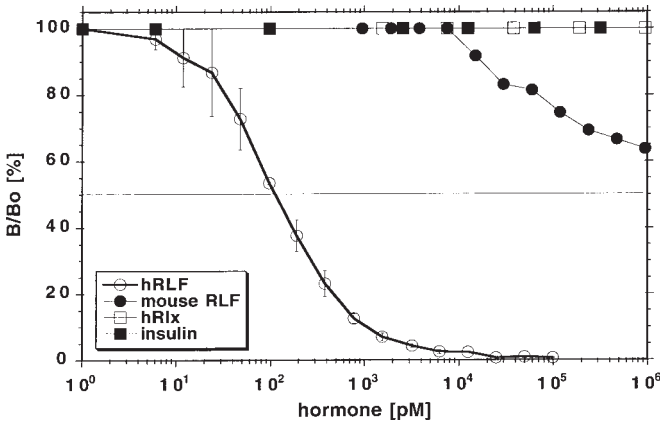

Fig. 1. RIA of hRLF, human relaxin, insulin, and mouse RLF using rabbit antihuman RLF antibodies and ^{125}I-RLF tracer. All hormones were diluted in PBS in the presence of 1% BSA and 0.01% sodium azide. All data points were collected in duplicate, and two assays were averaged. The bars indicate standard errors.

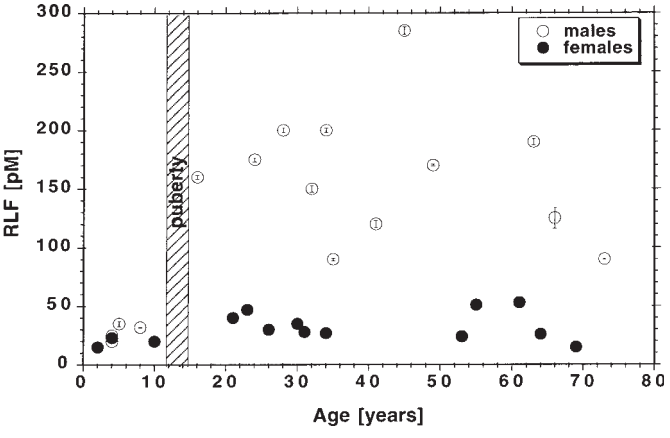

Fig. 2. RLF concentrations in human sera as a function of age and sex. Sera of 7 children (4 boys and 3 girls), 11 post-puberty females, and 12 postpuberty males were tested. RLF levels of two boys and one girl at the age of 4 were almost identical. Two data points were collected for each female serum and three data points for each male serum. The bars indicate standard errors.

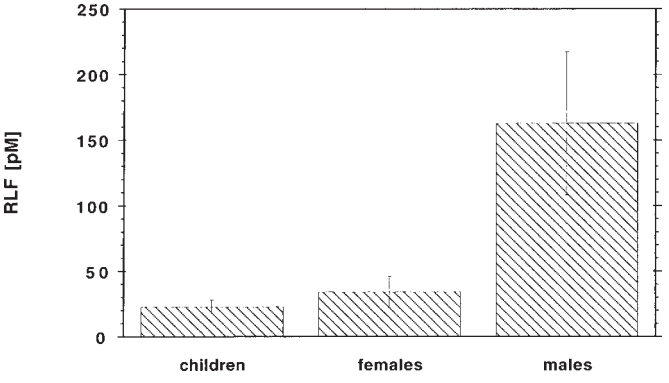

Fig. 3. Average RLF levels in human sera. The different groups comprise the average p*M* concentration in 100 µL serum of 7 children (4 boys and 3 girls), 11 postpuberty females, and 12 postpuberty males. The bars indicate standard deviation.

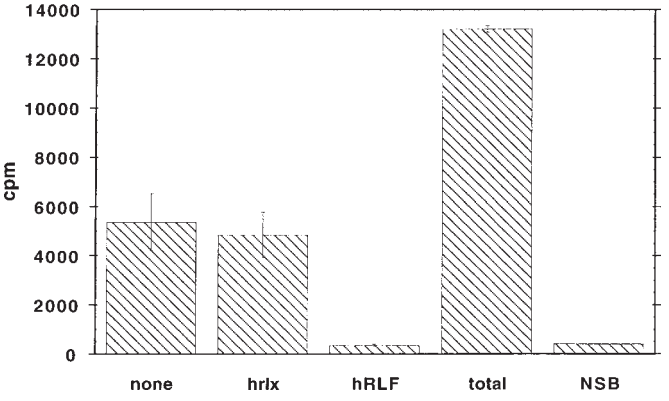

Fig. 4. Binding of the antibody to ^{125}I-RLF in human serum. Serum of three males with high RLF levels were pooled, and data were collected in duplicate. None: serum only; hRlx: addition of 600 ng/mL human relaxin; hRLF: addition of 600 ng/mL human RLF. Binding of the antibody to ^{125}I-RLF in the absence of serum: total: total binding; NSB: nonspecific binding. The bars indicate standard deviation.

tant to determine if it is a blood-borne factor, i.e., a hormone in both males and females before and after sexual maturity. Our RIA on about 30 sera of various age groups strongly suggest that the circulating RLF values change in response to the changing hormone levels at puberty. Once established, the RLF levels do not appear to change significantly with age in either gender. The fact that RLF stays at its immediate postpuberty level throughout life would suggest a more general role for the newly identified hormone. It is of interest to note that the blood levels of RLF in males are significantly higher than the peak values of relaxin in females in the first trimester *(11,12)*.

Gene silencing experiments in mice indicate that males without a functional RLF gene are sterile because of premiotic arrest of the sperm maturation process *(10)*, but gene knockout female mice remained fertile. The possibility of a relaxin support function *(8)* would provide a plausible mode of action for RLF in the female. Now that specific antibodies are available, progress can be expected from comparative studies of serum RLF levels in various human disorders. Although the jury is still out on other functions of RLF, the presence of significant numbers of uterine receptors makes one wonder about a possible role in females.

Materials and Methods

Human RLF *(8)*, human relaxin *(13)*, and mouse RLF were chemically synthesized, and the homogeneity and integrity of the structure verified. Porcine insulin was a gift from Eli Lilly. ^{125}I-RLF-mono-oxide was produced by iodination of a syn-

thetic precursor of hRLF and isolated by reversed-phase HPLC as described *(8)*. Protein concentrations were determined by UV spectroscopy using the following specific absorbance coefficients: 1.31 for hRLF, 0.92 for mouse RLF, 2.19 for human relaxin, and 1.05 for porcine insulin.

Antibodies to synthetic human RLF were raised in female white albino rabbits. The antigen was injected in complete Freund's adjuvant (5 μg/animal) sc over the haunches. Booster injections were given in incomplete adjuvant after 2 wk, followed by monthly injections over 6 mo. Antibody titers against synthetic RLF were determined periodically beginning 1 mo after the first injection. The final titer of the antibody used for RIA was 1:10,000.

RIAs were performed in PBS buffer pH 7.5 containing 1% BSA and 0.01% NaN_3. Standard curves were obtained using 100 μL of various concentrations of RLF, 100 μL of ^{125}I-RLF-mono-oxide (20,000 cpm), and 100 μL of antibody at a 1:10,000 dilution incubated for 16 h at 4°C. The soluble RLF/antibody complex was precipitated with goat antirabbit IgG antibody covalently bound to CNBr-activated cellulose *(14,15)*. About 1 mg of the corresponding IgG (Antibodies Incorporated) linked to 10 g of granular cellulose (Whatman F-11) was suspended in 1 L of 50 m*M* phosphate buffer (pH 7.5) containing 0.1% Tween 20. The efficiency of the carrier-bound IgG was predetermined. In standard experiments, 300 μL of the slurry were added to the assay, and the suspension shaken for 1 h at room temperature. After addition of 3 mL 50 m*M* phosphate buffer, pH 7.5, containing 0.1% Tween 20, the solid was collected by low-speed centrifugation. The pellet was washed once with the same buffer, the supernatants discarded, and the pellet counted in a γ-counter.

Nonspecific binding, as determined with hRLF at a concentration of 600 ng/mL (100 n*M*), was below 5%, but total binding was usually 75% of the counts added.

Human sera were obtained under standard conditions, but without anticoagulants from normal individuals varying in age from 2 to 73 yr. All samples were submitted to us under code, assayed, recorded, and returned to our collaborator, who then decoded them. Each individual sample was measured three times.

To determine the recovery of hRLF in human serum, the serum of postpuberty females was used. Synthetic hRLF was added to the serum to a 100-n*M* initial concentration. After serial dilution with human serum, the assay was performed as described previously.

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