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CLINICAL INVESTIGATION

Circulating luteinizing hormone receptor inhibitor(s) in boys with chronic renal failure

LEO DUNKEL, TANELI RAIVIO, JARMO LAINE, and CHRISTER HOLMBERG

Children's Hospital, University of Helsinki, SF-00290 Helsinki, Finland

Circulating luteinizing hormone receptor inhibitor(s) in boys with chronic renal failure. Patients with chronic renal failure frequently have hypogonadism. To elucidate the molecular mechanisms involved, we tested the ability of serum from these patients to inhibit recombinant human luteinizing hormone receptors. Using a cell line expressing functional human luteinizing hormone receptors, we found that adenosine 3′,5′-monophosphate (cAMP) production was markedly inhibited by sera from the patients, but not by sera from healthy subjects. Inhibition of cAMP production was associated with inhibition of ^{125}I-human chorionic gonadotropin binding. Inhibition of LH receptors by sera from patients correlated with the glomerular filtration rate and after renal allograft transplantation, decreased. Fractionation of serum samples indicated the receptor-inhibiting activity in proteins of molecular weights from 30,000 to 60,000 Daltons. When characterized and purified, the factor responsible may well be a new LH receptor antagonist of clinical significance.

In chronic renal failure (CRF), various endocrine axes, including the hypothalamo-pituitary-testicular axis, are susceptible to significant pathophysiological alterations [1, 2]. In uremic boys, bone age is delayed and pubertal maturation is often impaired or arrested, and in uremic men, clinical hypogonadism, consisting of diminished libido and potency and impaired fertility, may develop despite otherwise effective peritoneal dialysis or hemodialysis.

Alterations at both the pituitary and gonadal levels have been implicated in the pathophysiology of this condition in animal [3–8], and human [9–11] studies. Recent investigations evaluating pulsatile luteinizing hormone (LH) secretion have shown a reduced frequency of plasma LH pulses, implying the existence of a hypothalamic abnormality in uremia [6, 8, 11]. Functional impairment of the pituitary-gonadal axis may also be due to deficient Leydig cell steroidogenic function, since in uremic rodents [3, 5] and in men [9, 12] the testicular response to LH/hCG tends to be reduced, and reduced circulating LH bioactivity has been reported in chronic renal failure [9].

This observed impairment of steroidogenic capacity prompted us to study whether the serum of patients with CRF contains a factor capable of interacting with recombinant human LH receptors *in vitro*, which would explain the hypogonadism. Using a human cell line expressing functional LH receptors, we found that patients with CRF have circulating receptor inhibitors capable of interacting with recombinant human LH receptors by inhibiting adenosine 3′,5′-monophosphate (cAMP) production. This novel receptor inhibitor could explain the impaired Leydig cell androgen biosynthesis and hypogonadism seen in chronic renal failure. This finding raises the possibility of characterizing a naturally occurring substance with unique LH receptor-blocking ability.

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Methods

Nine boys with chronic renal failure due to various causes were studied (Table 1). The patients were studied at three different stages of their disease: in the first examination, they were having conservative treatment for CRF (7 patients, 9 samples); in the next stage they had developed end-stage renal disease, which was treated with continuous ambulatory peritoneal dialysis (CAPD; 8 patients, 9 samples); and the last examination took place after the patients had received a renal allograft (8 patients, 8 samples). The diseases leading to end-stage renal disease were nephronophthisis and reflux nephropathy due to congenital urethral valve, both in three patients, and congenital nephrotic syndrome, polycystic kidney disease and post-streptococcal glomerulonephritis, all in one patient each. Post-renal transplantation blood samples were drawn a mean time of nine months after transplantation (range 1 to 18 months). Seven of the nine patients received antihypertensive medication at the of blood sampling (all sampling times included). All except two of these patients received the same type of drug (calcium channel blocker or α-receptor blocker) both before and after transplantation. The two patients received a calcium channel blocker only after transplantation. Fifteen age-matched prepubertal boys, with no evident endocrine abnormality, were studied as controls (Table 1). The study was approved by the Ethics Committee of the Children's Hospital, University of Helsinki, Finland.

Reagents and hormones

Purified hCG (CR-127; 14,900 IU/mg) was obtained from the National Hormone and Pituitary Distribution Program, NIDDK, NIH. Purified human LH (WHO IRP 68/40) and 3-methyl isobutylxanthine were purchased from Sigma Co. (St. Louis, MO, USA). Cyclic AMP antibodies and ^{125}I-cAMP were purchased from ICN Immunobiologicals (Costa Mesa, CA, USA).

Gonadotropin immunoassays

Plasma LH and FSH levels were measured by time-resolved immunofluorometric assays (IFMAs), using reagents from Wallac (Kabi Pharmacia, Turku, Finland) as previously described [14].

Table 1. Clinical data of the patients with chronic renal failure according to the stage of the disease

	On conservative treatment	On continuous ambulatory peritoneal dialysis	After renal allograft transplantation	Controls
Number of patients	7	8	8	15
Age *years*	7.4 ± 1.3	7.2 ± 1.2	8.9 ± 3.0	7.7 ± 1.9
Pubertal stage[a]	G1P1	G1P1	G1P1	G1P1
Serum LH *IU/liter*	0.8 ± 0.6	0.9 ± 0.7	0.5 ± 0.3	0.4 ± 0.4
Serum FSH *IU/liter*	1.0 ± 0.4	1.0 ± 0.4	1.1 ± 0.4	0.9 ± 0.5
Glomerular filtration rate[b] *ml/min/1.73 m²*	15.0 ± 3.4	9.3 ± 0.7	72.9 ± 4.8	

Values are means ± SE.
[a] According to Tanner [13]
[b] According to Schwartz et al [27]

The LH standards were calibrated against WHO IRP 68/40, and FSH standards against the second IRP of pituitary FSH/LH (78/549). The assay sensitivity was 0.019 IU/liter for LH and 0.014 IU/liter for FSH as defined by the mean ± 2 SD of 12 replicates of a zero sample. Intra-assay coefficients of variation (CVs) were 2.5% and 2.1% (midrange), and interassay CVs were 4.5% and 4.1% for LH and FSH, respectively.

Expression of recombinant human LH receptors

The cDNAs coding for the entire human LH receptors were inserted into the pCMX expression vectors [15]. A human fetal kidney cell line (293) was used for the expression of the receptors. The cells were transiently transfected with the expression plasmids, using the calcium phosphate precipitation method [16].

Human LH receptor-based bioassays

For determination of cAMP production, LH receptor-expressing cells (2×10^5/tube) were treated with varying concentrations of purified hCG and LH (WHO IRP 68/40) as standards or with duplicate serum samples at three dose levels (3, 10, and 30 μl). The cells were then cultured for 18 hours in Dulbecco's minimal essential medium (DME; Gibco, Santa Clara, CA, USA) containing 5% calf serum in the presence of 0.25 mM 3-methyl isobutylxanthine. At the end of culture, extracellular cAMP levels were determined by specific RIA [17]. Intra-assay and inter-assay CVs of the cAMP RIA were 4.5% and 9.3% (midrange), respectively. The assay sensitivities was 3.0 IU/liter, as defined by mean ± 2 SD values of cAMP production by 12 replicate of non-treated cultures for the human LH receptor-based LH bioassay. LH and hCG standards showed parallel dose-dependent stimulation of cAMP production in the assays (data not shown).

Radioligand receptor assay

Iodination of hCG (CR-127) was performed using the lactoperoxidase method, as previously described [18]. The ^{125}I-hCG tracer was characterized by measuring its specific activity (100,000 cpm/ng) and maximal binding (45%) in a radioligand receptor assay using rat testicular membranes [19]. Non-specific binding was determined by adding a 1000-fold excess of unlabeled ligand (Pregnyl; Organon, Oss, The Netherlands). LH receptor binding in transfected 293 cells was performed by incubating the cells (2×10^5/tube) with sera for two hours at 22°C, after which ^{125}I-hCG was added. After incubation for another 18 hours, the samples were diluted fivefold with ice-cold Dulbecco's PBS (2 ml; DPBS) supplemented with 0.1% BSA and centrifuged at 800 × g for 15 minutes at 4°C. The pellets were washed twice with DPBS and, after the supernatant was discarded, were measured for radioactivity with a gamma counter.

Preliminary characterization of the inhibitor

For estimation of the molecular size of the inhibitor, sera from five subjects with CRF, showing high levels of LH receptor inhibiting activity, were fractionated using Centricon microconcentrators (Amicon, Danvers, MA, USA), with sizing limit at molecular weights of 10,000, 30,000, 60,000 and 100,000. After each fractionation step, serum was recovered into the initial volume by adding culture medium. Each of the serum fractions thus obtained was used to test the activity of LH receptor inhibitor in human LH receptor-based bioassay by measuring cAMP production. The protein nature of the stimulator was also tested by incubating sera from five subjects with CRF, showing high LH receptor inhibiting activity, with 5 μg/ml of trypsin (Sigma) at 37°C for 18 hours, followed by addition of 50 μg/ml of soyabean trypsin inhibitor. Since we had found that the inhibitor has molecular size larger than 30,000 Daltons, we separated trypsin (24,000 Daltons) from the putative inhibitor (30,000 to 60,000 Daltons) using Centricon microconcentrators with a molecular weight size limit of 30,000 Daltons before the assay.

Immunocytochemistry

Since the lower binding of radiolabeled hCG to human LH receptor expressing cells preincubated with sera from patients with chronic renal failure could have resulted from decreased receptor expression, the following immunocytochemical method was used to study receptor expression. An antibody was raised in rabbits to a synthetic peptide corresponding to amino acid residues 194-207 within the predicted extracellular domain of the human LH receptor. For immunocytochemistry, the antibody was specified as follows. There was minimal or no staining with replacement of the first antibody with equivalent dilutions of preimmune IgG or by preabsorption of the first (specific) antibody with the peptide against which it was raised. Furthermore, on Western analysis of membrane proteins from 293 cells transfected with the human LH receptor cDNA, a band with a mol wt compatible with that of the glycosylated human LH receptor protein was stained with the antibody. This band was abolished when the first antibody was replaced with preimmune serum or peptide preabsorbed first antibody, providing additional evidence for the specificity of the antibodies.

All the incubations were performed at 37°C in a humid chamber on 293 cells (transfected and non-transfected) affixed to glass slides. After blocking of the nonspecific biding sites with full porcine serum for 30 minutes at RT, the slides were incubated with sera obtained from control subjects or patients with chronic renal failure for three hours, followed by three washes with PBS (10 min each). After this the slides were incubated with a polyclonal antiserum to an extracellular portion the human LH

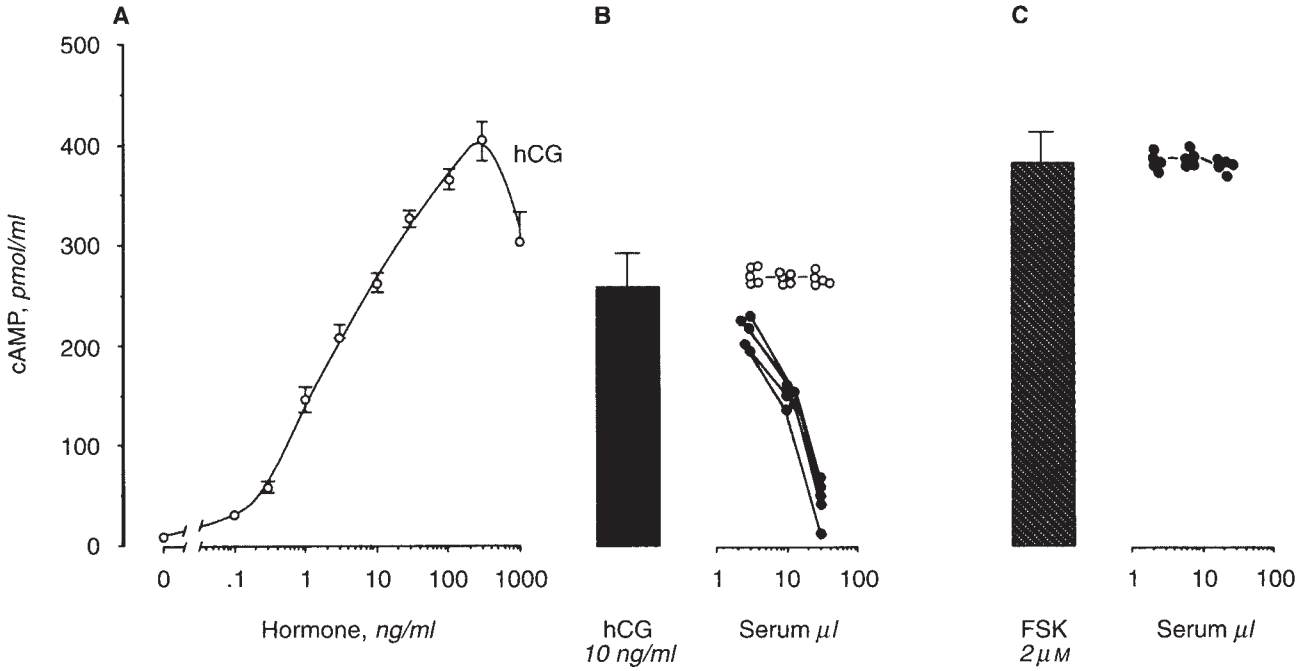

Fig. 1. *Inhibitory effect of serum taken from five representative patients in end-stage renal failure on cAMP accumulation in 293 cells expressing human LH receptors.* Extracellular cAMP accumulation was measured after incubation of the transfected cells (**A**) in the presence of hCG standard, (**B**) in the presence of a near-saturating concentration (10 ng/ml) of hCG, and in the absence or presence of three different amounts of serum taken from patients with chronic renal failure (●) or from control subjects (○), (**C**) in the presence of 2 mM of forskolin (FSK) and in the absence or presence of three different amounts of serum taken from patients with chronic renal failure (●). Data are means of 4 cultures.

receptor diluted 1:100. The slides were washed three times (10 min each) with PBS, followed by incubation for 1.5 hours with FITC-conjugated goat immunoglobulins to rabbit immunoglobulins (containing 5% porcine serum in PBS). After washing, the slides were viewed in fluorescence microscopy.

Statistical analysis

Results are presented as the mean ± SE of three to four replicate determinations. The analysis of variance (ANOVA) followed by Scheffe's F-test or Student's *t*-test was used, when appropriate, to determine significance of differences between mean values [20]. For all statistical analysis between different groups of patients, only one sample per patient was used. For Lineweaver-Burk plot analysis, bound and free hormone concentrations were calculated and their inverse values analyzed by linear regression analysis [21].

Results

Plasma levels of immunoreactive LH

Immunoreactive LH levels in serum samples from boys with chronic renal failure were similar to levels in normal boys at all stages of their disease (Table 1).

Serum LH receptor inhibitor(s) in boys with chronic renal failure

In the bioassay based on cAMP production by cells expressing human LH receptors, sera from the boys with chronic renal failure showed substantial inhibitory activity preventing cAMP accumulation (Fig. 1B). In contrast, all normal boys had undetectable LH receptor-inhibiting activity (Fig. 1B). To insure that the observed failure of cAMP to accumulate was indeed due to inhibition of the LH receptors on cAMP production, the effect of patient sera was also studied after stimulating cells expressing LH receptors with forskolin, a direct adenylyl cyclase activator. In these cells, cAMP production was not inhibited by the sera of the patients (Fig. 1C). This finding confirmed that the inhibition of cAMP production by sera of boys with chronic renal failure was mediated by human LH receptors.

The inhibition of cAMP production in cells expressing LH receptors by the sera of the patients was strongly dependent on the stage of the disease. Inhibition of cAMP production was strongest when patients were either receiving conservative treatment or being treated with peritoneal dialysis (Fig. 2A). After the patient had received a renal allograft, the level of inhibition was relatively low, indicating that the inhibitory effect of the patient's serum is at least partly a reversible phenomenon. Furthermore, the inhibition of cAMP production correlated inversely with the glomerular filtration rate (Fig. 2B).

Inhibition of ^{125}I-hCG binding in cells expressing human LH receptors by patient sera

Preincubation of cells expressing human LH receptors with sera from boys with chronic renal failure inhibited the binding of radiolabeled hCG in a dose-dependent manner (Fig. 3). At the highest dose of serum (30 µl), the ^{125}I-hCG binding was only 16 ± 2% of the maximal binding when the cells were coincubated with sera from patients treated with peritoneal dialysis, 23 ± 4% with sera from patients treated conservatively. After renal allograft transplantation, inhibition of hCG binding was less strong; it was 79 ± 5% of the maximal binding when the cells were coincubated with sera from patients of this group (Fig. 3). In contrast, none of

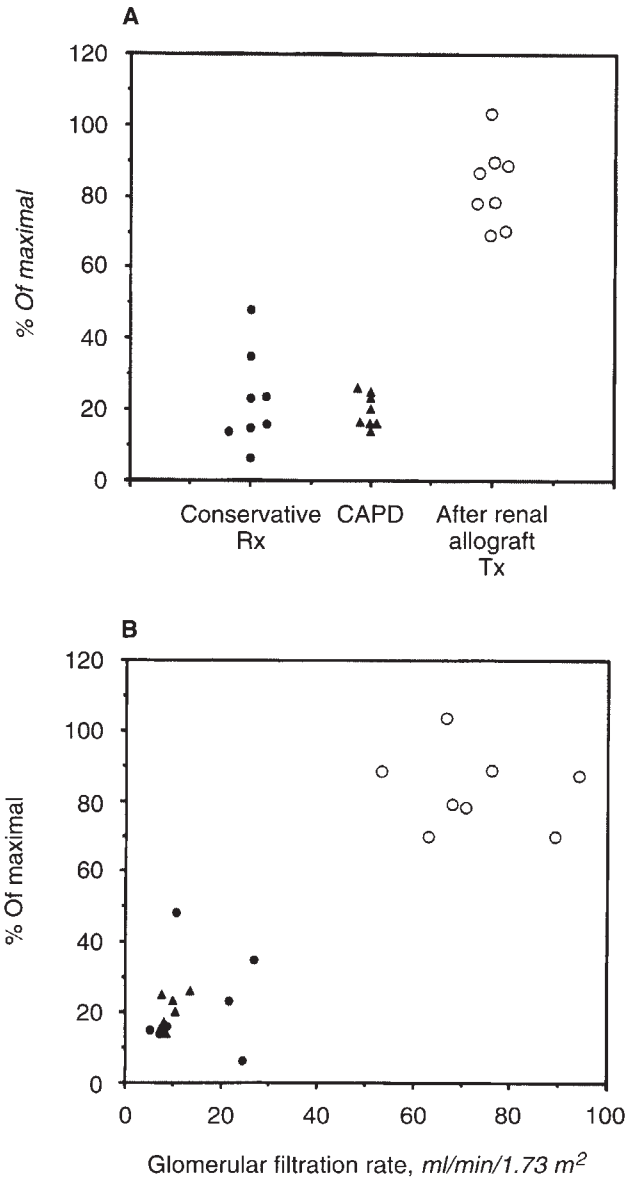

Fig. 2. *Inhibition of camp production in LH receptor-expressing cells by serum taken from patients with chronic renal failure (CRF).* The cells were simultaneously coincubated with a near-saturating concentration (10 ng/ml) of hCG and patient sera. Inhibition of camp production by the largest amount of patient serum used (30 μl) is expressed as a percentage of the cAMP production by hCG alone. A lower percentage indicates stronger inhibition (**A**) at various stages of the disease and (**B**) in relation to the glomerular filtration rate. Abbreviations are: Tx, transplantation; Rx, treatment; CAPD, CRF treated with continuous ambulatory peritoneal dialysis. Data are means of 4 cultures. Symbols are: (●) Cons Rx; (▲) CAPD; (○) after Tx.

the serum samples from healthy children inhibited binding of radiolabeled hCG. The difference in inhibition of ^{125}I-hCG binding between the healthy boys and the boys with chronic renal failure was significant ($P < 0.01$–0.001; Fig. 3B). The inhibition of the ligand binding to LH receptors by the patient serum correlated inversely with the glomerular filtration rate (Fig. 3C).

Since the putative LH receptor inhibitor appeared to interact with the ligand-binding domain of the receptor, we performed a saturation analysis to further clarify inhibitor-receptor interactions. After incubation with increasing concentrations of labeled hCG, a dose-dependent increase of specifically bound ^{125}I-hCG was detected in cells expressing human LH receptors, whereas no specific binding was observed in nontransfected cells (data not shown). Coincubation with varying amounts of patient sera inhibited ^{125}I-hCG binding in a dose-dependent manner (Fig. 4). Sera taken from patients with chronic renal failure appeared to reduce the total number of available binding sites. Kinetic analysis using Lineweaver-Burk plots showed that coincubation of patient sera and ^{125}I-hCG decreased maximal binding of ^{125}I-hCG ($P < 0.005$) without significantly affecting the apparent K_d value (Table 2, Fig. 4GB). These results indicate that the inhibition of ^{125}I-hCG binding by patient sera was non-competitive.

Molecular size and proteineous nature of the LH receptor inhibitor in sera of patients with CRF

To estimate of the molecular size of the serum factor inhibiting human LH receptors in patients with CRF, sera from five patients were separated using microconcentrators into fractions consisting of molecular weights ranging from less than 10,000 to above 100,000 Daltons. As shown in Figure 5, virtually all serum inhibitory activity based on cAMP production could be found in a fraction consisting of molecular weights 30,000 to 60,000. Also, treatment with trypsin decreased the LH receptor stimulating activity in serum by 96% (cAMP production before treatment with trypsin 231.9 ± 23.3 pmol/ml; after treatment with trypsin 9.3 ± 1.5 pmol/ml; $N = 7$), suggesting its proteineous nature.

Analysis of potential inhibition on LH receptor expression

To ensure that the decreased binding of radiolabeled hCG and decreased cAMP production were not due to decreased expression LH receptors we evaluated the LH receptor expression using immunocytochemistry. Figure 6 shows similar expression of LH receptors in cells preincubated with either control or uremic serum, indicating that the results obtained in the hCG binding experiments were indeed due to non-competitive inhibition of hCG binding.

Discussion

The present study demonstrates the presence of circulating inhibitors of human LH receptors in patients with chronic renal failure. This factor has a molecular weight between 30,000 and 60,000 Daltons and is believed to be responsible for the impaired Leydig cell steroidogenesis associated with this condition.

In the testis, Leydig cells are the only cells containing LH receptors and capable of androgen biosynthesis. After activation by specific ligands, LH receptors increase adenylyl cyclase activity by interacting with membrane-associated G proteins. The resulting increase in cAMP ultimately leads to an increase in androgen synthesis and secretion. Because of the difficulty in obtaining human testis cells, we used cells expressing recombinant human LH receptors and measured cAMP production. The assay systems closest to ours are modifications of rodent interstitial cell LH bioassays, which are widely used to measure LH bioactivity in serum and urine. However, the results obtained in LH bioassays with rodent interstitial cells are not necessarily comparable to an assay using recombinant human LH receptors, since there are

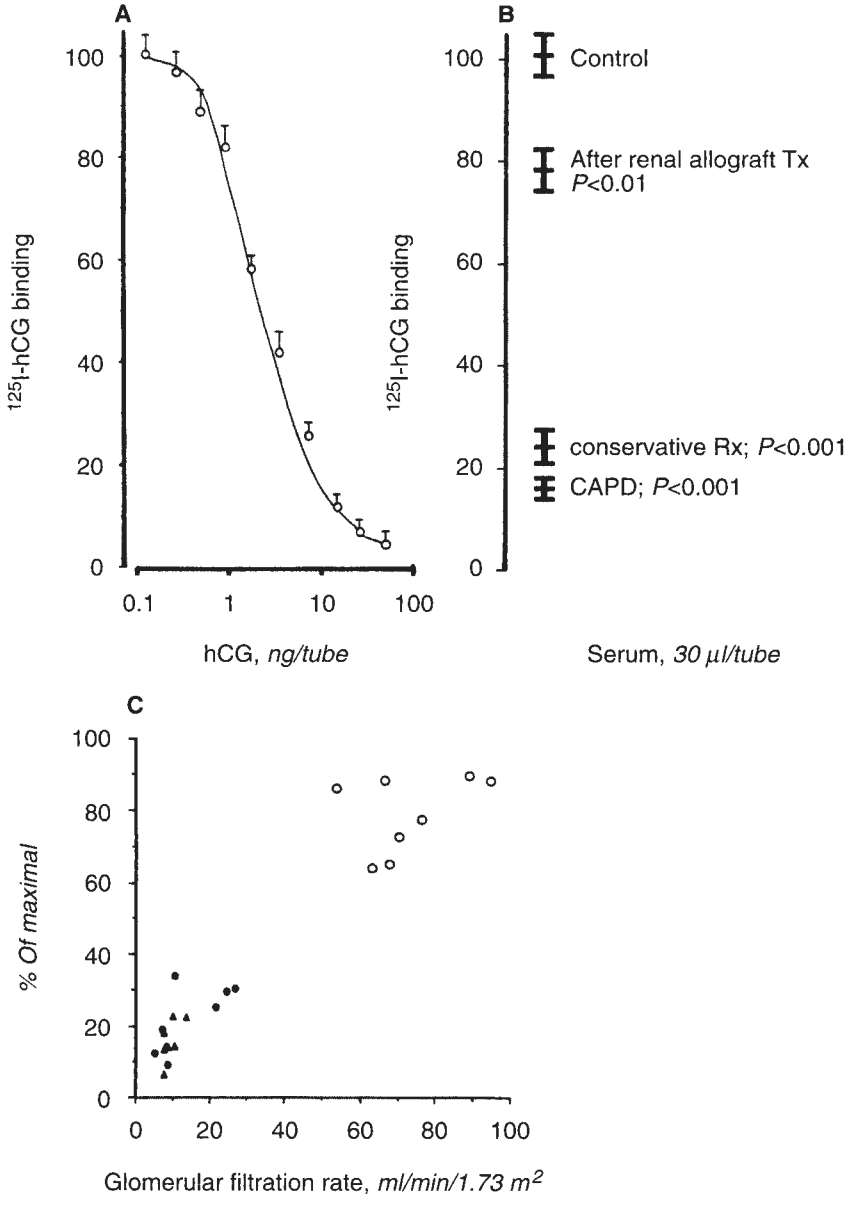

Fig. 3. *Displacement of ^{125}I-hCG binding to human LH receptors by hCG or by serum taken from boys with chronic renal failure (CRF) or from healthy boys.* Cells (2×10^5/tube) expressing human LH receptors were incubated for 18 hours after simultaneously adding ^{125}I-hCG with and without varying amounts of hCG (**A**) or 30 µl of patient serum (**B**). For hCG, displacement of ^{125}I-hCG is shown as a percentage of the maximal binding with each amount of unlabeled hormone, whereas for patient serum, displacement of ^{125}I-hCG is shown as a percentage of the maximal binding only for the highest amount (30 µl) of patient serum used. Results (mean ± SE) are shown for each group of boys, and the significance of difference from healthy children is indicated. (**C**) The same data in relation to the glomerular filtration rate; each data point represents one individual. Abbreviations are: Control, healthy boys; Tx, transplantation; Rx, treatment; CAPD, CRF treated with continuous ambulatory peritoneal dialysis. Symbols are: (●) Cons Rx; (▲) CAPD; (○) after Tx.

clear species differences between rodent and human LH receptors. For instance, recombinant human LH receptors, unlike their rodent counterparts, are highly species-specific in ligand recognition, further emphasizing the importance of evaluating ligand-receptor interactions and signal transduction mechanisms in a system using human LH receptors [15].

We have previously found that deglycosylated recombinant hCG binds to recombinant human LH receptors in a non-competitive manner, but is incapable of stimulating cAMP production, resulting in antagonism of LH action [22]. In the present study, we found that serum from boys with chronic renal failure had a similar effect on recombinant human LH receptors as that previously reported for deglycosylated hCG. The possibility that chronic renal failure affects the glycosylation process of pituitary hormones resulting in antagonism of receptor action by the naturally occurring receptor agonists remains to be investigated.

In addition to the observed antagonism at the LH receptors, serum from patients with chronic renal failure has been shown to decrease growth hormone and prolactin binding in rat liver membranes [23] and also to decrease the stimulation of SO_4 uptake by hypophysectomized rat costal cartilage *in vitro* [24], suggesting that growth hormone, prolactin, and insulin-like growth factor receptors are also down-regulated in chronic renal failure. Yet another impairment in receptor function and expression has been reported in low-density lipoprotein receptors in lymphocytes from uremic patients [25]. Although the molecular mechanisms underlying these changes in plasma membrane-bound receptor functions are unclear at present, the clinical

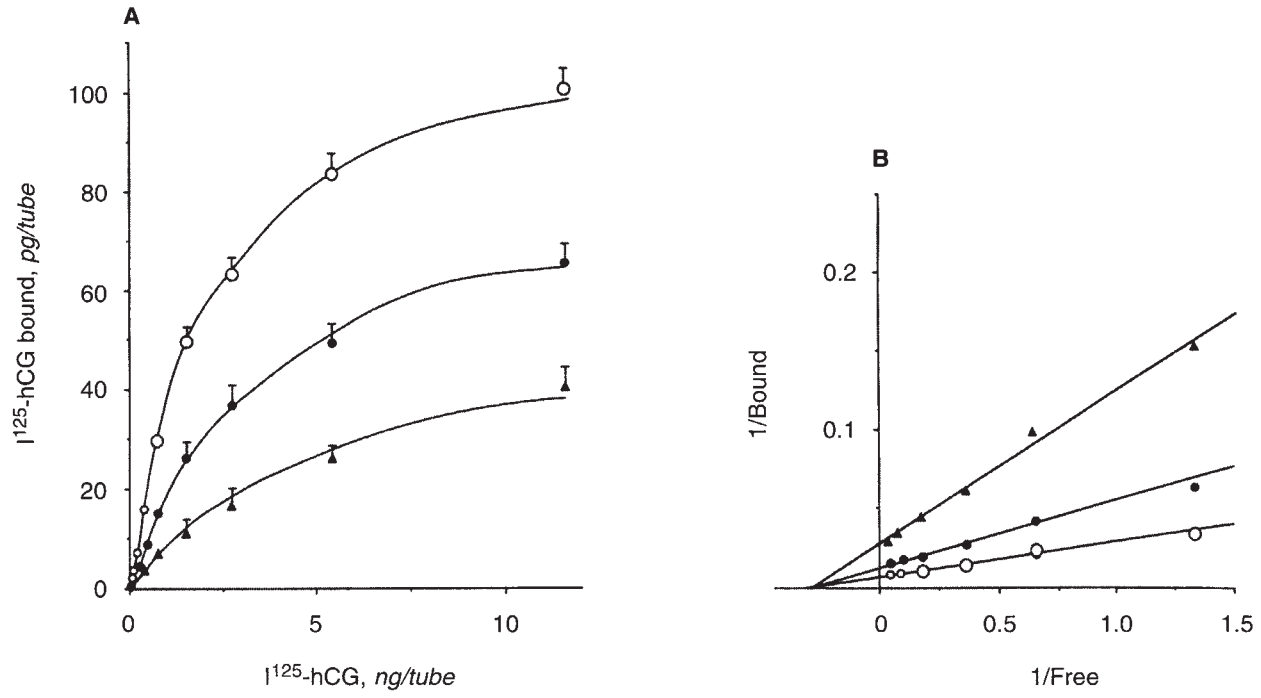

Fig. 4. *Competition for ^{125}I-hCG binding to human LH receptors by serum from a patient with chronic renal failure.* Cells (2×10^5/tube) expressing human LH receptors were incubated with increasing concentrations of ^{125}I-hCG in the absence or presence of different amounts of patient serum. Levels of specifically bound ^{125}I-hCG (**A**) are shown together with the derived Lineweaver-Burk plot (**B**). Symbols are: (○) no serum; (●) serum 3 μl/tube; (▲) 9 μl/tube.

Table 2. Effect of coincubation with patient serum and hCG on the maximal binding and dissociation constant (K_d) of ^{125}I-hCG binding to recombinant human LH receptors

Co-incubation with patient serum	Human LH receptor	
	Maximal binding *pg/tube*	Apparent K_d *10^{-10} M*
0 μl/tube	121.0 ± 3.1	2.3 ± 0.05
1 μl/tube	109.1 ± 3.4	2.1 ± 0.04
3 μl/tube	72.6 ± 2.5[a]	2.2 ± 0.06
9 μl/tube	39.0 ± 2.4[b]	2.8 ± 0.15

Human LH receptor-expressing cells (2×10^5/tube) were incubated with increasing concentrations of ^{125}I-hCG in the absence or presence of varying concentrations of patient serum as indicated. Serum and radiolabeled hormones were added simultaneously. Values are means ± SE. Lineweaver-Burk analysis was used to derive apparent K_d and maximal binding values. Results of three separate experiments are included.

[a] $P < 0.01$, [b] $P < 0.005$, significantly different from the group not coincubated with patient serum

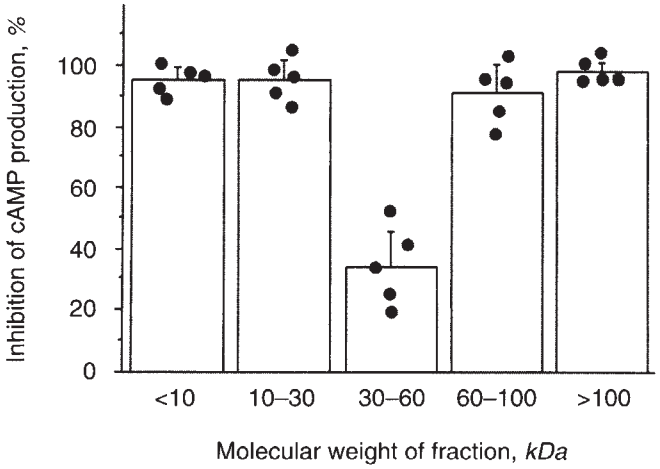

Fig. 5. *Estimation of the molecular size of circulating inhibitor of LH receptors in patients with CRF.* Serum from five patients with CRF were fractionated using Centricon microconcentrators followed by the analysis of the inhibitory activity on cAMP production. The cells were simultaneously coincubated with a near-saturating concentration (10 ng/ml) of hCG and patient sera. Inhibition of camp production by the largest amount of patient serum used (30 μl) is expressed as a percentage of the cAMP production by hCG alone. A lower percentage indicates stronger inhibition. Numbers under the bars indicate the range in molecular weights in kDa. Results are individual and mean ± SE values.

consequences are evident. Down-regulation of GH and insulin-like growth factor receptors are probably associated with poor growth in children [23, 24], and decreased low-density lipoprotein receptor expression and function likely result in hyperlipoproteinemia [25] in chronic renal failure.

Evidence for hypothalamic-pituitary involvement in the hypogonadism associated with chronic renal failure was recently obtained in uremic rats, which were found to have decreased rates of GnRH and LH secretion and decreased frequency of LH, but not of GnRH secretory pulses [8]. Similar alterations have also been reported in men with chronic renal failure [11]. The involvement of the hypothalamo-pituitary system can be explained by a circulating LH receptor inhibitor, since LH receptors are expressed in the central nervous system [26], and receptor inhibition

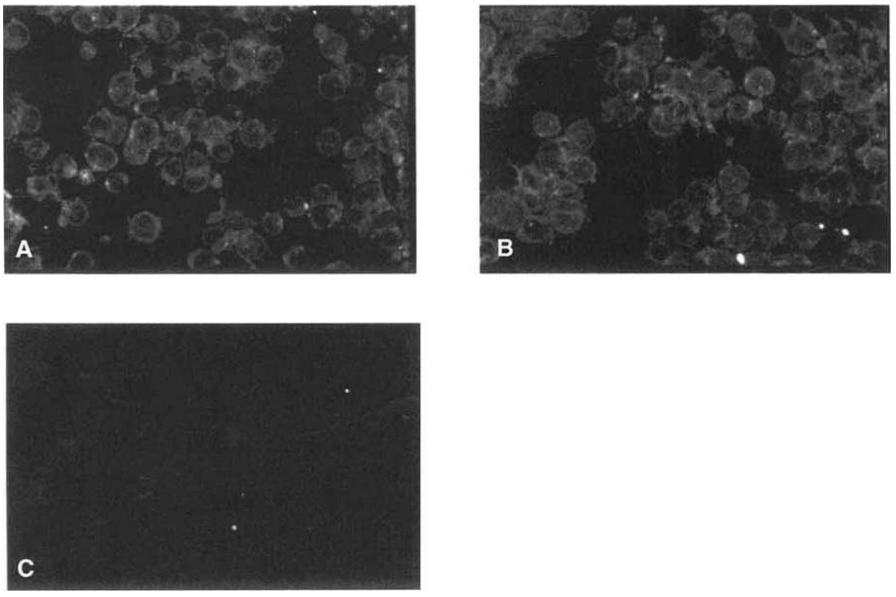

Fig. 6. *Identification of LH receptor expression in 293 cells transfected with human LH receptor cDNA.* The cells were preincubated with uremic serum (**A**) or control serum (**B**) followed by immunocytochemical identification of LH receptor protein (details are in the Methods section). Panels A and B show similar receptor expression. For the negative control, non-transfected 293 cells were used (**C**). These cells were treated as in panel B. Reproduction of this figure in color was made possible by a grant from Pharmacia Upjohn Oy, Finland.

may therefore be expected to result in modulation of the GnRH pulse generator.

Serum samples from boys with chronic renal failure inhibited receptor binding of ^{125}I-hCG, suggesting that the serum inhibitor may interact with the ligand-binding domain of the human LH receptor. Further biochemical characterization of the receptor inhibitor should allow the identification of a novel naturally occurring antagonist of LH receptors.

The LH receptor-inhibiting factor in CRF appears to be a large protein with molecular weight between 30,000 and 60,000 Daltons. This molecular weight range excludes the possibility that the receptor stimulating factor is an intact immunoglobulin molecule. Furthermore, the molecular weight range excludes the possibility of any toxic effects on the assay system caused by drugs used for treatment of CRF.

In conclusion, we have demonstrated that patients with CRF have a circulating factor capable of interacting with recombinant human LH receptors by inhibiting cAMP production. This factor may be involved in the pathogenesis of delayed sexual maturation in these patients. The recognition of novel receptor inhibitors in chronic renal failure was made possible by the new recombinant human LH receptor-based bioassay, which may also provide a useful tool for the diagnosis of other forms of hypogonadism. Furthermore, this assay may raise new possibilities for monitoring the extent of the functional gonadal involvement in the patients. Characterization and purification of this factor will provide a new antagonist for human LH receptors.

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Reprint requests to Dr. Leo Dunkel, Children's Hospital, University of Helsinki, FIN-00290 Helsinki, Finland.
E-mail: leo.dunkel@sci.fi

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