Gene expression and tissue concentrations of IGF-I in human myometrium and fibroids under different hormonal conditions

Katarina Englund¹, Bo Lindblom², Kjell Carlström¹, Inger Gustavsson¹, Peter Sjöblom¹ and Agneta Blanck¹,3

¹Department of Obstetrics and Gynecology, Huddinge University Hospital, Karolinska Institute, S-141 86 Huddinge, Stockholm and ²Department of Women’s and Children’s Health, Section for Obstetrics and Gynecology, Uppsala University, S-751 85 Uppsala, Sweden
3To whom correspondence should be addressed: Department of Obstetrics and Gynecology, K 57, Huddinge University Hospital, S-141 86 Huddinge, Sweden. E-mail: agneta.blanck@obgyn.hs.sll.se

The expression of insulin-like growth factor-I (IGF-I) was measured at the mRNA and protein level in myometrium and fibroids from women with and without preoperative treatment with a gonadotrophin-releasing hormone (GnRH) agonist for 3 months, from post-menopausal women, from pregnant women and in myometrium from women without fibroid disease. Women with menstrual periods were classified according to the phase of the cycle. In tissues from non-treated premenopausal women, IGF-I mRNA expression was significantly higher in fibroids than in myometrium, with no differences related to phase of the menstrual cycle. In post-menopausal women and in GnRH agonist-treated women responding to treatment, similar mRNA expression was seen in myometrium and fibroids but the concentrations were lower than in untreated premenopausal women. The IGF-I mRNA value in fibroids from pregnant women was higher than in any other group and myometrium from pregnant women exhibited higher mRNA expression than myometrium from non-treated premenopausal women. The IGF-I protein was more abundant in fibroids than in myometrium of non-treated premenopausal and of pregnant women and in both tissues the concentration was significantly higher in the group of pregnant women. The IGF-I protein concentrations in fibroids and myometrium from GnRH agonist-treated and post-menopausal women were similar to those from premenopausal non-treated women. High sex steroid concentrations in pregnant and non-pregnant women of fertile age seem to be associated with a higher expression of IGF-I in fibroids than in myometrium, suggesting that IGF-I contributes to the selective growth advantage of these tumours.

Key words: fibroids/hormone/human/IGF-I/myometrium

Introduction

Uterine fibroids, or leiomyomata, are the most common tumours in the female genital tract. They are benign and although many never give rise to symptoms, these tumours may cause bleeding disturbances, pelvic discomfort or pain and, occasionally, recurrent abortion and infertility. The circulating concentrations of oestrogen and progesterone are considered to be the major determinant for fibroid growth as these tumours stop growing and decrease in size after menopause. The size of fibroids (and of the myometrium) can be reduced by down-regulation of ovarian oestrogen and progesterone secretion with gonadotrophin-releasing hormone (GnRH) agonists (Friedman et al., 1987). The average decrease in volume of the fibroids following this treatment has been reported as >50% whereas the effect on total uterine volume is less pronounced (Puzigaca et al., 1994). Antiprogestins have been shown to decrease the size of fibroids to a similar degree (Murphy et al., 1993). During pregnancy, fibroids often increase in size during the first two trimesters, whereas very few continue to grow during the last trimester and most fibroids even shrink (Lev-Toaff et al., 1987). The reason for this lack of growth response, despite high serum concentrations of sex steroids during late pregnancy, is unknown.

When trying to explain the selective growth advantage of fibroids, several investigations have focused the interest on factors that are differentially expressed in fibroids and surrounding myometrium (for review, see Andersen and Barbieri, 1995). Although some of these investigations support the concept that both hormone receptors and growth factors might be important for the differences in growth regulation, so far no consistent hypothesis for this interaction has been presented. The insulin-like growth factors (IGFs) constitute a family of polypeptides with insulin-like and growth promoting activities (Humbel, 1990). They are produced not only in the liver but also in several extrahepatic tissues, including the uterus. Several studies indicate that both IGF-I and IGF-II are expressed and secreted in both fibroids and adjacent myometrium (Rein et al., 1990; Giudice et al., 1993; Vollenhoven et al., 1993). In the rat uterus, the expression of IGF-I (but not of IGF-II) has been shown to be regulated by oestrogens (Norstedt et al., 1989). In humans, the hormonal influence on expression is less clear, although a down-regulation of both IGF-I and IGF-II by GnRH agonists has been suggested (Rein et al., 1990). Previous studies indicate that membrane preparations from fibroids have more high affinity binding sites for IGF-I than myometrium, whereas no such difference could be seen for the binding of
Ultrasound measurements were performed in GnRH agonist-treated women. Dilutions of extracts from immediately frozen in liquid nitrogen and stored at −20°C. Pregnancy, GnRH agonist treatment, and after the menopause.

Materials and methods

Patients and treatments

A total of 59 patients were included in the study. None of them received any sex steroid or corticosteroid medication. The study was approved by the ethical committee of Huddinge University Hospital, Sweden, and a full and informed consent was obtained from all patients participating in the study. Fibroids and/or myometrial tissue were collected from 18 untreated premenopausal women, from 13 premenopausal women treated with a GnRH analogue (Goserelein; Zoladex®; Astra-Zeneca, UK) for 2–3 months before surgery, from five post-menopausal women and from five pregnant women. Of the latter group, one underwent hysterectomy at 12 weeks gestation since large fibroids made a regular abortion impossible, whereas the other four were subject to Caesarean section at term. From pregnant women only two paired samples of myometrium and fibroid(s) were collected. Myometrium only was collected from seven premenopausal and five post-menopausal women operated for other gynaecological diseases, not involving uterine enlargement and at Caesarean section from seven pregnant women without fibroids. In all, 66 different fibroids and 36 myometrial samples from 41 women with fibroid disease were analysed with respect to IGF-I mRNA expression, whereas the corresponding analysis of the IGF-I protein was performed on 26 fibroid supernatants. The number of fibroids per woman did not differ between groups.

Menstrual cycle pattern and last menstrual period (LMP) were noted for all women of fertile age and information about the response to treatment in terms of amenorrhoea or decreased bleeding was collected in GnRH agonist-treated women. All post-menopausal women except one had reached menopause >6 years ago and their post-menopausal status was confirmed by high FSH concentrations. Ultrasound measurements were performed in GnRH agonist-treated women before treatment and a few days after surgery. Fibroids and myometrial tissue were collected immediately at hysterectomy or at enucleation of fibroids. Tissue for mRNA and IGF-I analysis was immediately frozen in liquid nitrogen and stored at −70°C. Serum samples were collected between 07:00 and 08:00 on the morning of surgery and stored at −20°C.

Histopathological analysis

Tissue for routine histopathological examination was collected, fixed in buffered formaldehyde solution and embedded in paraffin. Sections were stained with haematoxylin and eosin and histopathological examination was performed to classify the lesions and to study the endometrium with respect to the phase of the menstrual cycle.

mRNA analysis

Total nucleic acids (TNA) were prepared according to Durnam and Palmer (1983) and TNA concentrations were determined by spectrophotometry. The DNA content was measured by fluorimetry (Labarca and Pagen, 1980) to certify the RNA/DNA-ratio. IGF-I mRNA values were determined by hybridization of TNA in solution as previously described (Durnam and Palmier, 1983). A complementary RNA (cRNA) probe was synthesized using Riboprobe system (Promega, Falkenberg, Sweden) with a 775 bp fragment of the gene coding for IGF-I as template (kindly provided by Professor Gunnar Norstedt, Centre for Molecular Medicine, Karolinska Institutet, Karolinska Hospital, Stockholm, Sweden). All samples were analysed several times. The results presented represent a typical experiment where all samples in duplicate have been hybridized with the same probe within a week. The background in terms of cpm/vial without TNA, expressed as percentage of input values, was 2–4%. At least double background concentrations were reached, within a range where a linear relationship between increasing amount of TNA and cpm/µg TNA was achieved. We found that the RNA content varies due to differences in endocrine conditions, probably reflecting differences in rate of proliferation between the different groups. For this reason we have chosen to relate IGF-I mRNA in all samples to their DNA concentrations, which is a more stable parameter than RNA or TNA content when proliferation differs.

Assay of IGF-I and sex steroids

Total IGF-I concentrations in tissue and in serum were determined by radioimmunoassay with a commercial kit from Nichols Products Corporation (San Juan Capistrano, CA, USA). Tissue specimens (100–200 mg) were cut from the frozen biopsies with a prechilled scalpel, weighed and cut into smaller pieces. The minced tissue was transferred to a prechilled (dry ice) capsule containing a tungsten ball. The capsule was frozen in liquid nitrogen and thereafter shaken at 1600 oscillations/min for 30 s in a dismembranation apparatus (Retsch, GmbH, Haan, Germany). This procedure was repeated once after intermediate freezing in liquid nitrogen. The pulverized tissue was then transferred to a prechilled tube and suspended in 10 volumes of buffer containing 10 mmol/l Tris, 1.5 mmol/l EDTA and 5.0 mmol/l Na2MoO4 (sodium molybdate) adjusted to pH 7.4 with HCl. The suspension was centrifuged at 24000 g for 30 min at +4°C and the supernatant was transferred to a prechilled tube and frozen at −70°C.

IGF-I was extracted from tissue homogenate and from serum with acid ethanol, as suggested by the manufacturer of the radioimmunoassay kit. The concentrations are expressed in µg of the World Health Organization (WHO) First International Reference Reagent IGF-I/87/518 (WHO, 1988). The detection limit in serum using the standard procedure suggested by the manufacturer was 0.6 µg/l and the intra- and interassay coefficients of variation were 6 and 10% respectively. Serial dilutions of extracts from fibroid and myometrial tissue yielded dilution curves parallel to the standard curve. Total protein in the tissue homogenates was determined by the Biuret method (Lowry et al., 1951).

Serum concentrations of oestradiol 17β, progesterone and FSH were determined by enhanced luminiscence competitive immunoassay using commercial kits (Amerset®; Amershams International, Little Chalfont, Bucks, UK). Serum FSH concentrations are expressed as IU/l of the Second WHO FSH IRP 78/549. Detection limits and within and between assay coefficients were 13.5 and 14.8% for 50 pmol/l oestradiol; 11.1 and 16.9% for 0.35 nmol/l progesterone; and 5.0 and 7.6% for 0.5 IU/l FSH respectively. In the statistical calculations values below the detection limits were set to 25 pmol/l for oestradiol and to 0.20 nmol/l for progesterone respectively.

Statistical analysis

A mean number of 1.8 fibroids per woman with fibroid disease was analysed. The mean value of 1–6 fibroids was calculated for each patient and used for further statistical analysis. The Kruskal-Wallis test was used to clarify whether differences between groups were
IGF-I in myometrium and fibroids

Table I. Patient characteristics and serum hormone concentrations (data are presented as means with ranges shown in parentheses). Controls represent patients without uterine disease, where only myometrium was collected

<table>
<thead>
<tr>
<th>Patient category</th>
<th>No. of patients</th>
<th>Age (years)</th>
<th>Parity (no. deliveries)</th>
<th>17β-oestradiol (pmol/l)</th>
<th>Progesterone (nmol/l)</th>
<th>FSH (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>7</td>
<td>41.6 (31–50)</td>
<td>2.1 (0–4)</td>
<td>331.2 (126–588)</td>
<td>7.8 (0.5–46.4)</td>
<td>8.9 (2.5–19.0)</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>5</td>
<td>65.2 (54–74)</td>
<td>1.5 (0–2)</td>
<td>99.7 (69–134)</td>
<td>0.6 (0.2–0.9)</td>
<td>52.8 (30.8–54.6)</td>
</tr>
<tr>
<td>Pregnant</td>
<td>7</td>
<td>30.1 (24–38)</td>
<td>1.9 (1–3)</td>
<td>&gt;14 000</td>
<td>150</td>
<td>n.d.</td>
</tr>
<tr>
<td>Patients with fibroids:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferatory</td>
<td>11</td>
<td>42.8 (35–52)</td>
<td>1.6 (0–3)</td>
<td>626 (184–1595)</td>
<td>1.4 (0.5–4.1)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Secretory</td>
<td>7</td>
<td>42.6 (36–50)</td>
<td>2.1 (0–4)</td>
<td>367.8 (225–765)</td>
<td>22.9 (5.5–45.3)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>5</td>
<td>64 (53–69)</td>
<td>1.4 (0–3)</td>
<td>106 (25–195)</td>
<td>1.6 (0.4–2.9)</td>
<td>35.4 (26.5–46.7)</td>
</tr>
<tr>
<td>GnRH agonist responders</td>
<td>9</td>
<td>41.1 (34–50)</td>
<td>1.1 (0–4)</td>
<td>53.2 (25–95)</td>
<td>0.8 (0.2–1.6)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pregnant</td>
<td>5</td>
<td>36.8 (34–46)</td>
<td>1.2 (1–2)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

GnRH = gonadotrophin-releasing hormone; n.d. = not determined.

*Represents five patients in the proliferative phase and two in the secretory phase.

Results

Data concerning parity and serum concentrations of oestradiol and progesterone and FSH are given in Table I. Non-treated premenopausal patients with fibroids were subclassified into proliferative (n = 11) and secretory (n = 7) groups according to menstrual cycle phase, based on menstrual cycle day, last menstrual period, endometrial histology and serum oestradiol and progesterone concentrations. Patients treated with GnRH agonist (n = 13) were subclassified as ‘responders’ or ‘non-responders’, depending on clinical response in terms of decreased fibroid volume and on oestradiol concentrations. Nine of these women responded to treatment with serum oestradiol concentrations of <100 pmol/l and a measurable decrease in fibroid size, while four did not respond, either in terms of decreased oestradiol concentration or in terms of reduced fibroid volume. The GnRH agonist-treated patients had a lower parity than the other premenopausal patients and, in seven out of 13 patients, a myomectomy and not a hysterectomy was performed, because of a desire for preserved fertility.

No cycle phase-related differences in IGF-I were observed in untreated premenopausal women with fibroids, either at the mRNA level or in protein concentrations (Tables II and III). In the statistical analysis, we therefore considered the untreated ‘cycling’ patients of fertile age as one single group. Five control patients without fibroids were in the proliferative phase and two were in the secretory phase of the cycle and these women were also considered as one group in comparison with the other categories.

IGF-I mRNA values are shown in Table II and Figure 1. The IGF-I mRNA values in non-treated premenopausal women were significantly higher in fibroids than in myometrium. This difference was not present in GnRH agonist-treated and post-menopausal patients. GnRH agonist-treated patients not responding to treatment had higher IGF-I mRNA values in myometrium and in fibroids than the corresponding group of patients responding to treatment and the difference was significant in the myometrium. No further statistical comparisons were made for the group of non-responders. No tissue difference was observed in pregnant women, but the IGF-I mRNA value in fibroids was significantly higher than in fibroids from untreated premenopausal women. Myometrium from pregnant women with or without fibroids had higher mRNA expression than the corresponding groups of non-treated premenopausal women, but this difference was significant only in myometrium from controls without fibroids. The mRNA level in fibroids and myometrium from both GnRH agonist responders and post-menopausal patients was

Table II. mRNA expression of insulin-like growth factor-I (IGF-I) (c.p.m./µg DNA) in human myometrium and fibroids obtained during the proliferative and secretory phases of the menstrual cycle. Data are presented as medians with range in parentheses

<table>
<thead>
<tr>
<th>Phase of cycle</th>
<th>Myometrium</th>
<th>Fibroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferatory (n = 11)</td>
<td>111 (29–255)</td>
<td>158.3 (26–332)</td>
</tr>
<tr>
<td>Secretory (n = 7)</td>
<td>128 (78–192)</td>
<td>138 (39–264)</td>
</tr>
</tbody>
</table>

*Significantly different from the corresponding myometrium (Wilcoxon signed rank test; P < 0.05).

Table III. Tissue concentrations of insulin-like growth factor-I (IGF-I) (ng/µg protein) in human myometrium and fibroids obtained during the proliferative and secretory phases of the menstrual cycle. Data are presented as medians with ranges in parentheses

<table>
<thead>
<tr>
<th>Phase of cycle</th>
<th>Myometrium</th>
<th>Fibroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferatory (n = 6)</td>
<td>0.64 (0.54–1.66)</td>
<td>1.63 (0.81–3.58)</td>
</tr>
<tr>
<td>Secretory (n = 4)</td>
<td>0.75 (0.58–1.04)</td>
<td>1.28 (0.58–1.89)</td>
</tr>
</tbody>
</table>

*Significantly different from the corresponding myometrium (Wilcoxon signed rank test; P < 0.05).
Figure 1. mRNA expression (c.p.m./μg DNA) of insulin-like growth factor-I (IGF-I) in myometrium (M) and fibroids (F) from untreated premenopausal, post-menopausal, gonadotrophin-releasing hormone (GnRH) agonist-treated premenopausal and pregnant women. The GnRH agonist patients were subdivided as responders (resp) or non-responders (non-resp) dependent on whether or not they responded to treatment. Control myometrium (C) was collected from premenopausal, post-menopausal and pregnant patients without uterine disease. Individual observations are indicated for each group and the solid lines represent medians. *Significantly different from the corresponding myometrium (Wilcoxon signed rank test; P < 0.05). To compare myometrium and fibroids in post-menopausal and pregnant women, the standardized \( t \) -test was used. #Statistically different from the corresponding myometrium from untreated premenopausal women with fibroids (Mann–Whitney \( U \)-test; \( P < 0.05 \)). &Statistically different from control myometrium from untreated premenopausal women (Mann–Whitney \( U \)-test; \( P < 0.05 \)). $Statistically different from fibroids from untreated premenopausal women (Mann–Whitney \( U \)-test; \( P < 0.05 \)). %Statistically different from the myometrium from GnRH agonist-treated women responding to treatment (Mann–Whitney \( U \)-test; \( P < 0.05 \)).

Tissue concentrations of IGF-I in myometrium and fibroids from patients are given in Figure 2. Due to lack of sufficient material tissue IGF-I was measured only in a limited number of samples. The concentrations of IGF-I were higher in fibroids than in myometrium from non-treated premenopausal and in pregnant patients, whereas no such difference was seen in the GnRH agonist-treated or post-menopausal women. The IGF-I concentration in both fibroids and myometrium from pregnant women was significantly higher than in the corresponding tissues from the untreated premenopausal group. No significant difference in IGF-I was observed in fibroids or in myometrium from GnRH agonist-treated or post-menopausal patients, when compared with the corresponding tissues from untreated premenopausal patients. No correlations were found between tissue and serum IGF-I concentrations (data not shown).

A significant positive correlation between IGF-I mRNA and tissue IGF-I protein was observed in the total material of fibroids when analysing all the samples where both variables had been measured (\( r_s = 0.45, P = 0.026 \)). This association disappeared when fibroids from pregnant women were excluded. No significant correlation was seen for the myometria. A positive correlation was found between the serum oestradiol concentration and IGF-I mRNA expression in the myometrium in all groups (\( r_s = 0.66, P = 0.0006, n = 28 \)), except the pregnant women (where oestradiol concentrations were in another range) and a significant correlation was also observed in the fibroids (\( r_s = 0.61, P = 0.002, n = 27 \)). No significant correlation was observed between serum oestradiol concentrations and tissue IGF-I protein, either in fibroids or in myometrium.

Discussion

The present study demonstrates that uterine fibroids have higher IGF-I mRNA expression and higher tissue concentration of IGF-I protein than the corresponding myometrium in non-treated women of fertile age. Previous reports concerning tissue differences in IGF-I mRNA expression have been inconsistent, showing higher expression in fibroids than in myometrium (Hoppener et al., 1988; Boehm et al., 1990) or no difference between the tissues (Vollenhoven et al., 1993). These discrepancies might partly be due to the fact that fibroids are individual tumours where the expression of IGF-I varies considerably between fibroids from the same patient, which is
also the case for another endocrine related variable, i.e. sex steroid receptor contents (Englund et al., 1998). In the present study, up to a four-fold difference in mRNA expression was observed between fibroids from the same patient. This implies the importance to analyse more than one fibroid from each patient whenever possible.

The influence of ovarian sex steroids on IGF-I mRNA expression in fibroids and in myometrium is clearly demonstrated by the lower expression observed in both fibroids and myometrium from post-menopausal and GnRH agonist-treated women responding to treatment and the significant correlation between serum oestradiol and IGF-I mRNA values. It is also worth noting that protein differences were not present under these conditions, i.e. oestrogen-depletion. However, in these two groups the tissue concentrations of IGF-I remained high, although the lack of tissue difference was similar to the mRNA data. In contrast, no variation due to phase of the menstrual cycle was observed, either for IGF mRNA expression or for IGF-I protein. It has been suggested that the IGF-I mRNA expression in fibroids is influenced by oestrogen and enhanced during the late proliferative phase of the menstrual cycle (Giudice et al., 1993). This is supported by our findings of significant correlations between serum oestradiol and IGF-I mRNA levels in both myometrium and fibroids. The lack of menstrual cycle related variation suggests that a more detailed subclassification, with a larger patient material, may be necessary to further analyse this issue.

Another study (Vollenhoven et al., 1994) reported no differences in mRNA expression of IGF-I, IGF-II or the IGF binding proteins (IGFBPs), either in fibroids or in myometrium, from women pretreated with a GnRH agonist, compared with tissues from untreated women. Our data indicate that reduction of both fibroids and mean uterine volume in GnRH agonist-treated and post-menopausal women is associated not only with decreased concentrations of oestradiol and progesterone but also with decreased amounts of IGF-I mRNA. These observations suggest a role for IGF-I in growth regulation of both fibroids and myometrium.

The differences between fibroids and myometrium were more pronounced for tissue concentrations of IGF-I than for IGF-I mRNA. This may partly be explained by the fact that the capacity of membrane preparations to bind IGF-I is higher in fibroids than in myometrium, which may favour accumulation of IGF-I (Tommola et al., 1989; Chandrasekhar et al., 1992). The capacity to bind IGF-I is determined by the concentration of type I IGF receptors (Van der Ven et al., 1994) and by the availability of IGFBP, which are present in the uterus (Giudice et al., 1993). It is worth noting that there was a positive correlation between IGF-I mRNA and IGF-I protein only in fibroids in the present material, further suggesting the importance of receptors and IGFBPs as modulators of IGF-I availability.

In female mice, carrying a targeted deletion in the Igfl gene (Igfl null), a pronounced hypoplasia of the uterus was seen, especially in the myometrium (Baker et al., 1996). Following stimulation with oestradiol the mitotic index in myometrium from 20 day-old mice was extremely low in the Igfl-null mice compared with that in wild-type mice (Adesanya et al., 1999). The authors also presented data favouring the view that IGF-I is required for G2 progression in the oestradiol-induced mitotic cycle. These findings further support the concept that IGF-I has a key role in uterine growth control.

The down-regulation of IGF-I mRNA in post-menopausal and GnRH agonist-treated women is not reflected at the protein level. This discrepancy might suggest a post-transcriptional modification of IGF-I gene expression or indicate that the endocrine mechanisms involved in regulation of receptors and/or IGFBPs differ from those that determine IGF-I tissue concentrations. However, it is important to keep in mind that the total tissue content of IGF-I determined by the method used here by no means reflect the biological availability of the protein, as both bound and unbound IGF-I are measured.

The high IGF-I and IGF-I mRNA values observed in both fibroids and myometrium of term pregnant women is in accordance with previous observations (Stjernholm et al., 1996) where a four-fold higher amount of IGF-I mRNA was found in the cervix of term pregnant, compared with non-pregnant, women. It is presently difficult to say whether the observed increase in IGF-I expression is related to the stimulatory effect of high sex steroid levels during pregnancy or whether the high levels of placental growth hormone, especially during the second half of pregnancy, contributes (Caufries et al., 1993). However, the alterations may also be part of more specific responses of the uterus, in terms of, e.g. remodelling of connective tissue before parturition.

Taken together, the present study favours the view that IGF-I is involved in sex steroid-mediated growth regulation of both fibroids and myometrium in the human uterus. However, to be able to measure and evaluate the bioavailability and effects of the protein, further information about the receptors and binding proteins and their endocrine regulation is essential.

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