Changes in the glycosylation pattern of circulating gonadotropins after acute administration of gonadotropin-releasing hormone in patients with anorexia nervosa

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Abstract

To study the involvement of gonadotropin-releasing hormone (GnRH) in glycosylation of circulating gonadotropin isoforms in anorexia nervosa (AN), 14 amenorrhoic patients with AN, 14 age-matched volunteers in early follicular phase, and five normal-weight re-fed patients with AN were investigated under baseline conditions and after acute administration of GnRH. Plasma gonadotropins were assayed using IRMA before and after concanavalin A affinity chromatography. Baseline plasma gonadotropin levels were lower for both AN and re-fed AN patients than in controls (P<0.005). The increase in FSH and LH after GnRH administration was lower than in controls for AN (P<0.005) and re-fed AN (P<0.005 and P<0.05 respectively) patients. Percentages of total gonadotropin not bound to concanavalin A (complex carbohydrate chains) under baseline conditions were higher in patients with AN than in controls (P<0.005) but decreased after GnRH administration (P<0.001). In re-fed AN patients, the percentage of unbound FSH was higher than in controls (P<0.05), and decreased after GnRH administration (P<0.001), whereas the percentages of unbound LH were not significantly different from controls either before or after GnRH administration. These data suggest that: (a) the acute administration of GnRH induces quantitative and qualitative changes in circulating gonadotropin isoforms in both normal controls and AN patients; (b) during recovery the LH response in re-fed AN patients is associated with a glycosylation pattern that is the same as that for controls.

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Introduction

Anorexia nervosa (AN) is a complex psychiatric disorder, with increasing incidence especially among adolescent and young women, associated with multiple disturbances in central monoaminergic systems and endocrine abnormalities (1). Dysfunctions in the hypothalamus–pituitary–gonadal axis and amenorrhea are common features (2). Although a clear relationship exists between menstrual dysfunction, weight loss and malnutrition, amenorrhea can predate weight loss and may persist despite weight gain (2, 3). An abnormality in the central regulation of the gonadotropin-releasing hormone (GnRH) pulse-generation system has been described in both undernourished animal models (4–6) and humans with AN, with a reduced gonadotropin pulsatility and an age-inappropriate gonadotropin secretion pattern (3, 7–10). Pulsatile GnRH therapy in AN patients has been reported to induce the quantitative restoration of both episodic luteinizing hormone (LH) secretion and pituitary LH responsiveness to GnRH (11). However, the central mechanism underlying diet-induced hypogonadism remains unexplained and persists after the return to a normal diet (1–3, 6, 12).

The microheterogeneity of gonadotropin isoforms is well known in both physiologic and pathologic conditions (13–18). Similarly to thyrotropin-releasing hormone (19–23), a direct effect of GnRH on gonadotropin N-linked carbohydrate branching has been postulated to occur (17–18). Qualitative changes in both circulating follicle-stimulating hormone (FSH) and LH occur in amenorrheic AN patients, with evidence of circulating gonadotropin isoforms that are less sialylated (24). Although the effects of exogenous GnRH administration on gonadotropin isoform distribution pattern have been evaluated in animal models (6, 12) and humans under different conditions (25–28), no studies have been performed on patients with AN to evaluate whether the acute GnRH administration influences circulating gonadotropin glycosylation.

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The pattern of elution of plasma gonadotropins from concanavalin A (Con A) was therefore investigated in 14 amenorrhoic patients with AN and five normal-weight re-fed AN patients before and after acute administration of exogenous GnRH in comparison with 14 age-matched normally cycling women.

Materials and methods

Subjects and study procedure

Fourteen hospitalized females, aged 16–27 years, with AN and secondary amenorrhea lasting at least 6 months, and 14 age-matched volunteers of normal weight and with regular menstrual periods, some of whom had already enrolled in our previous study (24), were investigated. The AN patients weighed less than 70% of ideal body weight, with body mass index <17 (normal values [n.v.] 20–25 kg/m²). Ideal body weights were determined from the Metropolitan Life Insurance Co. tables of New York 1983 (29). Five re-fed AN patients, aged 19–31 years with body mass index >20, were included in the study; they had all attained normal eating behavior and a weight greater than 85% of ideal for at least 6 months, and two had resumed normal menstrual periods (three and five menses). All the patients were recruited from the Clinical Nutrition Unit of Federico II University; informed consent had been given in accordance with the Helsinki Declaration. Diagnoses were based on the Diagnostic and Statistical Manual of Mental Disorders (DSM IV) criteria (30). No patient or control was taking any medication known to affect ovary function or had received prior estrogen therapy. Re-fed AN patients and normal controls were evaluated in the precocious follicular phase (4–6 days after the onset of uterine bleeding).

GnRH tests were performed between 0800 and 1000 h after an overnight fast. Blood samples were obtained immediately before and 15, 45, 60 and 90 min after the i.v. injection of 100 μg GnRH (Relisorm L100; Serono, Roma, Italy), via an indwelling antecubital venous catheter. The samples were centrifuged and stored at −80°C until assayed.

RIA

Gonadotropins in both plasma and Con A-affinity column fractions were measured in duplicate using an ultrasensitive IRMA (FSH IRMA CT and LH IRMA CT; Radim, Pomezia, Italy; n.v. 2.5–13 and 1.4–13 IU/l respectively) employing a combination of two monoclonal antibodies: a tube-immobilized β-specific antibody and a labeled one specific for the intact FSH and LH dimers. The sensitivity of the IRMA (level at mean B₀±2.5 S.D.) was 0.1 IU/l for both FSH and LH. The intra-assay coefficients of variation for low and medium values were for FSH 5.1 and 2.7% respectively and for LH 2.8 and 1.5% respectively. Plasma levels of 17β-estradiol were assayed three times under basal conditions (4–6th day) by RIA using commercial kits (CIS; Tronzano Vercellese, Italy; n.v. 70–220 nmol/l).

Con A affinity chromatography

As previously reported (24), 1 ml aliquots of unextracted serum were added to 3 ml 50% (v/v) agarsbound Con A (Pharmacia Fine Chemicals, Milan, Italy) in PBS (0.01 mol/l phosphate and 0.15 mol/l NaCl, pH 7.4). They were subsequently placed on 10 ml columns, incubated at room temperature and shaken for 1 h. The FSH and LH fractions that did not bind to Con A (UB) were obtained after centrifugation for 30 min at 1000 g at 4°C. The Con A-bound FSH and LH fractions were obtained using the same buffer containing 300 mmol α-methyl manno pyranoside, with a total recovery of 85–98%. The column fractions were stored at −20°C until immunoassayed. Percentages of unbound FSH and LH (% FSH-UB and % LH-UB) were calculated as the ratio of FSH-UB and LH-UB to total FSH and LH concentrations.

Statistical tests

Plasma gonadotropin concentrations were log-transformed before statistical analysis to correct for the heterogeneity of variance. Statistical differences between baseline and post-treatment values were assessed by ANOVA between groups, followed by Newman–Keuls’ test and Student’s unpaired t-test. Differences at P<0.05 were considered statistically significant. Significant relationships between variables were analyzed using Pearson product-moment correlations.

Results

Plasma FSH and LH levels of AN patients (n = 14), re-fed AN patients (n = 5) and controls (n = 14) during baseline and GnRH-stimulated conditions are reported in Table 1. Maximum values for FSH and LH were obtained after 45 min (P=0.001). A significant difference is observed up to 90 min after GnRH administration (P<0.05), except for LH values for AN patients. For FSH and LH, mean values were significantly lower for both AN patients and re-fed AN patients than normal controls under baseline conditions (P<0.005). After acute GnRH challenge, the maximum values for FSH and LH were significantly lower for AN patients than for controls (P<0.005). The maximum FSH and LH values were also significantly lower for re-fed AN patients than for controls, although to a different significance (P<0.005 and P<0.05 respectively).

The mean values of % FSH-UB and % LH-UB after and before GnRH stimulation are reported in Table 2. The minimum values during the test, expressed as mean±S.D., were obtained after 45 min (P<0.001 except for % LH-UB for normal controls and re-fed AN
patients). A significant difference is observed up to 90 min after GnRH administration for both % FSH-UB and % LH-UB for AN patients, and for % FSH-UB only for re-fed AN patients (P<0.001). Under baseline conditions, the % FSH-UB values were significantly higher for both AN and re-fed AN patients than controls (P<0.005). The % LH-UB was also significantly higher in AN patients than controls (P<0.005), while the values for re-fed AN patients did not significantly differ from controls. After acute GnRH challenge, the minimum values for % LH-UB were significantly higher for AN patients than for controls at 45–90 min (% LH-UB values between re-fed AN patients). A weak negative correlation was found in normal controls between maximum FSH values and minimum % FSH-UB values at 45 min (r = −0.6).

Discussion

We previously found a significant increase in the percentage of FSH and LH molecules not retained on Con A (complex-type asparagine-linked oligosaccharides) in a group of amenorrhoic AN patients than in eumenorrhoic normal-weight controls, and we related this alteration to a decrease in gonadotropin bioactivity (24). In the present experiment, we have shown that, in spite of low plasma concentrations of stimulated gonadotropin, the glycosylation pattern of gonadotropins in AN patients is modified immediately after an acute GnRH challenge. In particular, in accord with previous reports (26, 27, 31), in normal controls a single acute GnRH dose significantly decreases % FSH-UB 45 min after GnRH injection, without any further decrease in the naturally occurring secretion of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Plasma FSH and LH concentrations after an acute challenge with 100 μg GnRH.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after GnRH (min)</td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>AN</td>
</tr>
<tr>
<td>0</td>
<td>8.7</td>
</tr>
<tr>
<td>(6.5, 12.0)</td>
<td>(2.0, 5.8)</td>
</tr>
<tr>
<td>15</td>
<td>12.2</td>
</tr>
<tr>
<td>(7.6, 20.9)</td>
<td>(2.9, 12.5)</td>
</tr>
<tr>
<td>45</td>
<td>17.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>(11.8, 24.1)</td>
<td>(4.9, 20.4)</td>
</tr>
<tr>
<td>90</td>
<td>14.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(9.2, 24.3)</td>
<td>(5.0, 17.1)</td>
</tr>
</tbody>
</table>

Values are geometric means and their 95% confidence limits for the concentrations.
NC, normal controls (n = 14); AN, patients with AN (n = 14); Re-fed AN, normal-weight re-fed AN patients (n = 5).
<sup>a</sup>P < 0.001 vs baseline; <sup>b</sup>P < 0.005 vs NC; <sup>c</sup>P < 0.05 vs NC; <sup>d</sup>P < 0.05 vs baseline.

**Table 2** Percentage of Unbound FSH and LH fractions (UB) after an acute challenge with 100 μg GnRH. Values are means ± so.

<table>
<thead>
<tr>
<th>Time after GnRH (min)</th>
<th>Plasma FSH (IU/l)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>AN</td>
<td>Re-fed AN</td>
<td>NC</td>
<td>AN</td>
<td>Re-fed AN</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>80.5 ± 9.3</td>
<td>96.7 ± 7.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.3 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.2 ± 7.0</td>
<td>74.4 ± 16.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.1 ± 5.4</td>
</tr>
<tr>
<td>15</td>
<td>71.7 ± 9.7</td>
<td>70.5 ± 17.2</td>
<td>75.6 ± 8.3</td>
<td>14.1 ± 5.5</td>
<td>51.6 ± 29.3</td>
<td>16.3 ± 2.7</td>
</tr>
<tr>
<td>45</td>
<td>65.2 ± 11.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.0 ± 11.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.3 ± 7.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.5 ± 3.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.5 ± 15.9&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>12.1 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>90</td>
<td>74.6 ± 9.2</td>
<td>64.1 ± 15.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.2 ± 6.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.4 ± 3.9</td>
<td>21.5 ± 14.1&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>13.2 ± 1.5</td>
</tr>
</tbody>
</table>

NC, normal controls (n = 14); AN, patients with AN (n = 14); Re-fed AN, normal-weight re-fed AN patients (n = 5).
<sup>a</sup>P < 0.001 vs baseline; <sup>b</sup>P < 0.005 vs NC; <sup>c</sup>P < 0.05 vs NC.
baseline unbound LH isoforms. Conversely, in AN
patients, a single acute GnRH dose significantly reduces
the abnormally high percentage of unbound FSH and
LH 45 min after GnRH injection, so that for FSH-UB the
significant baseline differences between AN patients and
controls are no longer evident, whereas for LH-UB they
are still present, although to a lower significance than
under baseline conditions. In other words, the intrapitu-
titary gonadotropin pools of patients with AN contain
higher amounts of mannose-rich less-sialylated mole-
cules after acute GnRH administration than before, and
these higher concentrations are the same as baseline
concentrations in control subjects in the follicular
phase, as previously reported to occur in thyrotropin
secretion after thyrotropin-releasing hormone adminis-
tration (19–21, 32). In AN, changes in the percentage
of unbound FSH and LH are still evident 90 min after
GnRH administration, whereas in normal controls the
percentage of unbound FSH returned to baseline values.
This may be related to a relative impairment of the
unstimulated pituitary to process the gonadotropin
molecules to more mature isoforms, as also described for
thyrotropin (19, 20). Conversely, in normal controls,
also taking into account the selective survival of serum
isoforms, the secretion of all available isoforms follows
the initial preferential release of bound isoforms 90 min
after GnRH. As naturally occurring FSH contains more
complex-type oligosaccharide chains than LH (31), the
effect of GnRH appears to be more striking on LH than
on FSH, particularly for AN patients.

Similarly to AN patients, re-fed AN patients exhibit
higher % FSH-UB values than normal controls, with a
significant decrease after GnRH administration; how-
ever, neither baseline nor stimulated % LH-UB values
were significantly different from normal controls. This
suggests that, in normal-weight re-fed AN patients, the
increase in basal plasma levels of estrogen is related to
the secretion of LH isoforms that are not different from
those of control subjects in the follicular phase,
probably because of restoration of the endogenous
GnRH pulse frequency. In this context, the changes in
baseline and stimulated LH glycoisoforms in re-fed
AN patients may provide a possible additional
marker for the prediction of the reversal of endocrine
abnormalities.

The microheterogeneity of pituitary and circulating
gonadotropin isoforms appears to be related to the
endocrine milieu (18). In particular, GnRH (25–27, 33)
and estrogens (12, 34–39) have been proposed to be
physiologic modulators of gonadotropin polymorphism.
Although a direct estrogen effect on the glycosyltrans-
ferases in the pituitary has also been postulated (40),
the relative roles of increased GnRH pulse frequency
and rising sex hormone concentration have not been
clarified (26). Previous data obtained in animal models
suggest that nutritional factors and percentage of body
fat are also relevant to the failure of the GnRH neuro-
secretory mechanism, with depression of gonadotropin
secretion independently of ovarian presence (4, 6). No
studies in humans have as yet provided clear informa-
tion on the relevance of nutritional status to the central
mechanism underlying diet-induced hypogonadism, in
particular in AN (2, 8, 9, 33). In this context, the study
of chronic undernourished AN patients, with low plasma
levels of endogenous estrogen and without estrogen
replacement therapy, represents a useful
approach for evaluating the effects of endogenous alte-
ration of pulsatile GnRH secretion on gonadotropin
isoform secretion. The present paper is the first to show
in humans a relationship between GnRH, amenorrhea,
nutrition and body weight, although their exact roles
remain to be defined (2, 3). Previous experiments have
been performed on prepubertal animal models before
the development of a mature reproductive system
dependent on exogenous nutritional manipulations. In
contrast, our studies were performed on reproducively
regressed adults, with fully established reproductive
neuroendocrine system and endogenous eating beha-
viour alterations. In spite of the metabolic and endocrine
differences between animal and human models, our
data confirm the effect of acute GnRH administration on
the glycosylation pattern of gonadotropins in AN
patients. In re-fed AN patients, the effect of GnRH
may be related to weight gain and increase in estrogens.
In AN, however, we cannot infer that the GnRH effect is
mediated through estrogenic influences, as changes in
gonadotropin isoforms are also observed in the presence
of low estrogen concentrations without interference
from estrogen replacement therapy. Finally, the involve-
ment of ovarian factors other than sex steroids, such as
inhibin, activin and follistatin, cannot be excluded from
the present study.

Although further evidence obtained with a larger
group of patients is required to confirm this hypothesis,
our data suggest that, as far as patients with AN are
concerned, nutritional state, body fat increase, GnRH
and estrogen concentrations are presumably only cofac-
tors in the pathogenesis of amenorrhea. This hypothesis
is in line with neuroendocrine dysregulation of gonado-
tropin secretion in AN being part of the overall
alteration of the monoaminergic neurotransmission
underlying the eating behaviour disturbances. In
conclusion, AN patients provide a suitable and original
model for evaluating the effects in vivo of GnRH
administration upon gonadotropin glycosylation pat-
tern. In these patients, the abnormalities in glycosyla-
tion of gonadotropin isoforms are reversed by acute
GnRH administration. The contribution of chronic
energy deprivation during refeeding needs to be further
investigated.

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