In vivo administration of a GnRH antagonist to male mice: effects on pituitary gonadotropin secretion in vitro

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Abstract. The potent luteinizing hormone-releasing hormone antagonist [N\text{-}Ac\text{-}D-p\text{-}Cl\text{-}Phe\textsuperscript{12},D\text{-}Irp\textsuperscript{5},D\text{-}Arg\textsuperscript{6},D\text{-}Ala\textsuperscript{8}]\text{GnRH} (4 mg/kg) was administered sc once or daily for 21 days to immune-deficient (nude) and normal immune-competent (NIC) male mice derived from the same genetic background. Effects of in vivo pretreatment with the antagonist on gonadotropin secretion from hemipituitary glands from both types of mice were studied in vitro in the presence or absence of synthetic GnRH. Treatment with the GnRH antagonist caused differential effects on release of FSH and LH from and amounts of FSH and LH in hemipituitary glands. Pituitary FSH secretion was effectively inhibited, whereas effects on pituitary LH were less evident or nonsignificant under these experimental conditions. Long-term treatment with the antagonist caused larger effects on pituitary secretion and content of FSH, when compared with short-term treatment. No significant effects of duration of treatment on secretion or pituitary content of LH were detected. Addition of synthetic GnRH to the incubation medium caused stimulation of gonadotropin release. Therefore, it was concluded that the high doses of this GnRH antagonist were not able to block GnRH receptors effectively in the pituitary glands of nude and NIC male mice. The incomplete suppression of LH secretion by this high dose of the GnRH antagonist may partly explain the inability of the antagonist to suppress plasma testosterone levels and the growth of androgen-dependent tumours in male mice.

The hypothalamic decapeptide luteinizing hormone-releasing hormone stimulates the secretion of follicle-stimulating hormone and luteinizing hormone from the pituitary gland. Since the isolation and characterization of GnRH, numerous highly potent GnRH analogues with agonistic or antagonistic properties have been synthesized. These compounds have been studied in a variety of species using both in vitro and in vivo systems (Grady et al. 1985; Labrie et al. 1980; Thau et al. 1985).

Acute administration of GnRH agonists to male experimental animals causes a prolonged release of gonadotropins, which stimulates Leydig cell function, resulting in increased plasma testosterone concentrations. In contrast, chronic administration of GnRH agonists results in suppression of both pituitary gonadotropin secretion and testicular testosterone production. This suppressive action of GnRH agonists is presumably due to down-regulation of the number of pituitary GnRH receptors and subsequently decreased gonadotropin secretion. However, the initial stimulation of secretion of gonadotropin, and therefore of testosterone during the initial phase of chronic treatment with GnRH agonists, is a clear disadvantage when the agonist is used therapeutically in the treatment of hormone-dependent tumours such as prostatic cancer. Use of GnRH antagonists, which block the action of endogenous GnRH and sup-

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press gonadal function in this way, might be indicated under these circumstances.

In an in vivo study on the effect of treatment of nude mice with a GnRH antagonist on the growth of a human transplanted prostatic tumour and on peripheral concentrations of gonadotropins and testosterone (van Steenbrugge et al. 1987), it was observed that the antagonist used was not able to suppress peripheral hormones permanently. In order to study the reason for this apparent noneffectiveness of the GnRH antagonist, we incubated pituitary glands from the same animals in vitro in the presence or absence of synthetic GnRH, starting at 2 and 24 h after injection of the GnRH antagonist. Since it has been reported that athymic nude mice have significantly reduced concentrations of both pituitary and plasma LH and FSH in comparison with heterozygous normal immune-competent (NIC) animals from the same breeding colony (Rebar et al. 1982), the pituitary responses after treatment with the antagonist in immunodeficient (nude) and NIC male mice were also compared.

Materials and Methods

Animals

Athymic male nude mice or NIC animals with a similar genetic background (Balb/c) were used. They were maintained under standard laboratory conditions as described earlier (van Steenbrugge et al. 1984).

GnRH and its peptide analogue

The antagonist [N-Ac-D-D-Cl-Phe12-D-Trp3-D-Arg8,D-Ala11]GnRH, Org 30276 (Coy et al. 1982) was synthesized and supplied by Organon International bv (Oss, The Netherlands). This peptide was used for in vivo treatment regimens.

Synthetic GnRH (Relefact®, Hoechst AG, Frankfurt am Main, Germany) was used in the in vitro studies.

Treatment of the animals

Male nude or NIC mice were treated with Org 30276 (4 mg/kg body weight) or vehicle for 21 days by means of daily sc injections. The animals were killed 2 h after the last injection. In addition, a group of NIC animals was killed 24 h after this treatment. The response of the pituitary gonadotropin secretion was also studied 2 h after a single dose of the antagonist.

Because the number of available nude mice was limited, the same vehicle-injected group was used as a control group for the 21-day and 1-day injected nude animals. For the NIC-mice, separate control groups were used; no significant differences were obtained for any of the parameters studied in these control groups.

Hemipituitary glands

The animals were exsanguinated from the orbital sinus under ether anesthesia and subsequently killed by decapitation. After removal of the neurohypophysis, the anterior pituitary gland was cut in two equal parts, which were placed immediately in separate glass counting vials containing 1 ml Medium 199 (Gibco, Grand Island, NY). After all pituitary glands were collected, medium was removed and replaced with fresh medium with or without addition of synthetic GnRH (1 mg/l). The hemipituitary glands were incubated for 4 h at 37°C in a thermostatic shaking water bath (frequency 140 cycles/min) in an atmosphere of 5% CO₂ in air. At hourly intervals, 200 μl aliquots were taken from the flasks and stored at −20°C until assayed for FSH and LH. After the incubations, the pituitary tissue was placed in phosphate-buffered saline (0.01 mol/l, pH 7.0), homogenized and stored at −20°C until assayed for FSH and LH content.

Hormone estimations

The concentrations of FSH and LH in pituitary culture medium and pituitary homogenates after incubation were measured using the radioimmunoassay technique as described by Welschen et al. (1975). All results are in terms of NIADDK-rat-FSH RP-1 or NIADDK-rat-LH RP-1. Hormones in plasma, media or pituitary gland from various treatment groups within one experiment were measured in one assay. Intra-assay variations were 11.6 and 13.3% for FSH and LH, respectively.

Statistical procedures

Data for secreted and pituitary tissue gonadotropins were processed by analysis of variance, comparing the effects of the antagonist, GnRH, strain of mice, time of the experiment, and interaction of the effects of antagonist treatment and the presence of GnRH in vitro, as indicated in the Results section. Differences were considered to be significant when P was < 0.05.

Results

Time-related release of LH and FSH from incubated hemipituitary glands

Typical results for the hourly estimations of LH and FSH in the incubation media of hemipituitary glands from NIC mice, treated with the GnRH antagonist for 21 days and killed 2 h after the last injection, are shown in Fig. 1a and 1b, respectively.

No significant differences could be detected between the secreted amounts of LH as a result of the pretreatment in vivo (P > 0.10), whereas the effects of GnRH were significant (P < 0.005). No interaction between the effects of in vivo antagonist treat-
ment and in vitro addition of GnRH could be detected.

In contrast, the effects of antagonist pretreatment on FSH release in vitro were significant (P < 0.005), whereas GnRH caused significant increases of FSH secretion (P < 0.005). Again, no significant interaction between the effects of the antagonist and GnRH was detected (P > 0.20).

Similar time patterns of LH and FSH release were obtained in all groups of incubations. For this reason, only the data for the secreted LH and FSH after 4 h of incubation will be discussed in the remainder of this paper, especially since this is the only time for which tissue levels of the gonadotropins are available.

**Influence of mouse strain on pituitary LH and FSH release and content**

Comparison of the amounts of LH and FSH, present in the medium or in the homogenates of the pituitary glands, indicated no significant differences between FSH and LH levels in the medium for the nude and NIC mice. Tissue content of both LH and FSH was slightly, but significantly lower in nude mice than in NIC animals (P = 0.009 and 0.003, respectively), when the data of all treatment groups were combined in the analysis of variance. However, these differences were small relative to the changes induced by the treatments. Therefore, data from both types of mice were combined for the further analysis of the data.

**Effect of a single injection of antagonist on pituitary LH and FSH release and content**

Data on the pituitary release and content of gonadotropins obtained when the pituitary glands were incubated for 4 h, starting 2 h after a single injection of the GnRH antagonist, are summarized in Table 1.

The release of both gonadotropins from control pituitaries was stimulated significantly by the addition of GnRH to the incubation medium (P = 0.038 for FSH, < 0.001 for LH). Pretreatment with the antagonist significantly affected the release of FSH from the pituitary glands (P = 0.038), whereas the effect on LH was not significant. Finally, the interactions between GnRH and antagonist treatments were significant for both FSH and LH (P = 0.001 and 0.024, respectively). Tissue content of LH and FSH was not significantly affected by the treatment.
with GnRH or antagonist alone. However, the interactions were again significant for both hormones (LH: $P<0.038$, FSH: $P=0.050$).

**Table 1.**

Release and content of LH and FSH (μg/hemipituitary) during and after 4 h incubation of hemipituitary glands from control and GnRH antagonist (Ant)-treated mice. The pituitary glands were collected 2 h after a single injection with the antagonist (4 mg/kg body weight), and incubated in the presence or absence of synthetic GnRH (1 mg/l).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSH</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Tissue</td>
</tr>
<tr>
<td>Control</td>
<td>2.3 ± 0.26</td>
<td>12.1 ± 0.96</td>
</tr>
<tr>
<td>Control + GnRH</td>
<td>5.2 ± 0.43</td>
<td>13.1 ± 0.85</td>
</tr>
<tr>
<td>Ant</td>
<td>2.7 ± 0.26</td>
<td>12.8 ± 1.43</td>
</tr>
<tr>
<td>Ant + GnRH</td>
<td>3.4 ± 0.28</td>
<td>10.0 ± 0.82</td>
</tr>
</tbody>
</table>

Results have been expressed as means ± SEM, N = 7–11. See text for significance of differences.

**Effect of 21 days pretreatment with the GnRH antagonist on the pituitary release and content of gonadotropins**

Results have been summarized in Table 2. Incubation of pituitary glands from mice, pretreated for 3 weeks with the antagonist, showed results which were different from those obtained after incubation following a single injection of the antagonist, as far as FSH was concerned.

In the incubations, starting 2 h after the last injection of the antagonist (Table 2A), FSH concentrations in the medium were affected significantly by GnRH and the antagonist, whereas the interaction was no longer significant at this time ($P<0.001$, $P<0.001$ and $P<0.22$, respectively). The amounts of FSH and LH in the cells were significantly reduced ($P<0.001$ and $P<0.003$, respectively) after in vivo treatment with the antagonist. Here, the influence of GnRH and the interaction were not significant.

LH concentrations in the medium were significantly stimulated ($P<0.001$) by the addition of GnRH, whereas pretreatment with the antagonist had no significant effect. Similarly, the interaction of antagonist and GnRH showed no significant effect.

When the incubation started 24 h after the last injection, no differences in any of the parameters could be detected between these results and those obtained starting the incubation 2 h after the last injection (Table 2B). Again, no significant interactions between the effects of GnRH and the antagonist were found. Antagonist treatment significantly suppressed FSH release and content ($P=0.001$ and 0.005, respectively), whereas the suppression of LH release and content was not significant ($P=0.092$ and 0.105, respectively). GnRH did not affect tissue levels of LH and FSH ($P=0.28$ and 0.54, respectively) or the release of FSH ($P=0.067$), but stimulated LH release significantly ($P=0.036$).

**Discussion**

This in vitro study shows that there is hardly any difference between pituitary release and content of gonadotropins in nude and NIC Balb/c mice during the incubations despite hormonal abnormalities present in vivo in the nude male mice. A slight but significant reduction in FSH and LH content of hemipituitary glands from nude male mice in comparison to NIC-mice was observed. These results, found after 4 h of incubation, support earlier data on reduction in gonadotropins observed in athymic nude male mice by Rebar et al. (1982).

The potent GnRH antagonist Org 30276 effectively inhibited the growth of hormone-dependent transplantable tumours in rats and in mice (Redding & Schally 1983; Schally et al. 1983). In addition, studies with mouse pituitaries in vitro (Kato & Sairam 1983) showed that lower doses of this antagonist caused a more effective suppression of LH than of FSH release, whereas the pituitary secretion of LH and FSH induced by maximally stimulatory doses of GnRH was inhibited. On the other
Table 2.
Release and content of LH and FSH (μg/hemipituitary) during and after 4 h incubation of hemipituitary glands from control and GnRH antagonist (Ant)-treated mice. The pituitary glands were collected 2 h (A) or 24 h (B) after the last of 21 daily injections with the antagonist (4 mg/kg body weight), and incubated in the presence or absence of synthetic GnRH (1 mg/l).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSH</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Tissue</td>
</tr>
<tr>
<td>A Control</td>
<td>2.6 ± 0.33</td>
<td>12.3 ± 0.97</td>
</tr>
<tr>
<td>Control + GnRH</td>
<td>4.8 ± 0.30</td>
<td>13.8 ± 1.03</td>
</tr>
<tr>
<td>Ant</td>
<td>0.9 ± 0.09</td>
<td>7.0 ± 0.39</td>
</tr>
<tr>
<td>Ant + GnRH</td>
<td>2.4 ± 0.17</td>
<td>7.0 ± 0.76</td>
</tr>
<tr>
<td>B Control</td>
<td>3.2 ± 0.22</td>
<td>14.9 ± 2.95</td>
</tr>
<tr>
<td>Control + GnRH</td>
<td>3.9 ± 0.45</td>
<td>12.4 ± 2.89</td>
</tr>
<tr>
<td>Ant</td>
<td>1.8 ± 0.33</td>
<td>6.9 ± 1.24</td>
</tr>
<tr>
<td>Ant + GnRH</td>
<td>2.5 ± 0.35</td>
<td>6.6 ± 1.02</td>
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</tbody>
</table>

Results have been expressed as means ± SEM, N = 7–11 in A, N = 5–6 in B. See text for significance of differences.

hand, it was shown that a relatively high dose of this antagonist affected neither the growth of the human hormone-dependent prostatic tumour PC82 nor the weights of testes and accessory sex organs in nude mice (van Steenbrugge et al. 1987). In nude as well as in NIH mice no significant suppression of the plasma levels of testosterone occurred 24 h after the last injection following daily administration of a high dose of the GnRH antagonist. Two hours after administration of a single dose of the antagonist, the basal release of LH and FSH was slightly affected, when compared with the controls, whereas the stimulatory effect of synthetic GnRH on the release of LH and FSH was significantly lower in the animals pretreated with the antagonist. These results correspond with plasma concentrations of gonadotropins measured in the same groups of animals 2 h after a single injection of the antagonist: plasma levels of LH were significantly suppressed, whereas the effect on FSH was not significant, probably because of the small number of samples in which FSH concentrations were estimated (van Steenbrugge et al. 1987).

Daily administration of a high dose of the GnRH antagonist suppressed pituitary content and release of FSH, when the tissue was collected 2 h after the last injection. The effect of antagonist treatment on the release of FSH was stronger than after a single injection of the antagonist (cf. Tables 1 and 2), probably as a result of the significantly decreased pituitary content of FSH. Furthermore, there was no longer a significant interaction between the effects of antagonist and GnRH. The LH content of the pituitary glands was also suppressed by pretreatment with the antagonist. However, the release of LH from the incubated pituitary glands was not different from that after a single injection of antagonist and the interaction effect between the actions of antagonist and GnRH, which was very clear after a single injection, was no longer present after pretreatment. This disappearance of interaction between the effects of GnRH and antagonist on the secretion of both LH and FSH suggests that the antagonist no longer prevents the action of GnRH during the 4-h incubation period after its repeated administration. The reason for this apparent disappearance of the effectiveness of the antagonist after repeated treatment remains unclear. Finally, the results obtained 24 h after the last injection of the antagonist were essentially not different from those obtained 2 h after the last injection. This contrasts with the in vivo findings,
where peripheral levels of LH were suppressed 2 h, but not 24 h after the last injection (van Steenbrugge et al. 1987).

Under the conditions used by Bex et al. (1982) and Wang et al. (1983) mice have been reported to be extremely resistant to the paradoxical inhibitory effect of agonistic analogues of GnRH on the pituitary gonadal axis, whereas it was shown that in rats, GnRH antagonists were effective to inhibit the growth of the homone-dependent Dunning rat prostate tumour (Schally et al. 1983). This difference may be due to differences in interactions between components of the hypothalamic-pituitary-gonadal axis in rats and mice. For instance, studies in male mice have demonstrated that, in contrast to the situation in rats, a rapid and sustained fall in pituitary GnRH receptors occurred after castration (Naik et al. 1984). Some authors suggested that in rats, the antagonist competitively inhibits the binding of GnRH to its pituitary receptors; in this was pituitary gonadotropin secretion is blocked (Heber et al. 1982; van Rees 1985).

Grady et al. (1985) have indicated that in rats, pituitary LH and FSH secretion shows differential dependence on GnRH in vivo: treatment with a GnRH antagonist caused a rapid and intense decline in LH secretion, whereas the effect on FSH is more gradual and less intense. These differences were not present under in vitro conditions. In mice, however, chronic administration of the antagonist causes suppression of the pituitary content and release of FSH rather than LH. The reason for these differences in response between rat and mouse remains unclear.

In conclusion, the dose of the GnRH antagonist in vivo was apparently sufficient to decrease pituitary FSH content and release significantly in this mouse model, whereas pituitary LH was less responsive to the inhibitory effect of the antagonist. This incomplete inhibition of LH release observed in mice indicates that this species is not suitable for studying the effects of GnRH antagonists on testicular testosterone and suppression of growth of hormone-dependent prostatic tumours.

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