Structure−Activity Studies of Reduced-Size Gonadotropin-Releasing Hormone Agonists Derived from the Sequence of an Endothelin Antagonist

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We have previously determined that Ac-D-Trp-Leu-Asp-Ile-Ile-Trp (peptide I), an endothelin antagonist, binds specifically ($K_i = 1.9\ \mu M$) to the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. Moreover, peptide I exhibits a GnRH agonistic activity, mediated directly by the GnRH receptor. We now report structure−activity studies of peptide I in respect to its interactions with the GnRH receptor. Our studies suggest that the bioactive conformation of peptide I, recognized by the GnRH receptor, is of a cyclic nature. Thus cyclic analogues of peptide I exhibit higher affinity to the GnRH receptor and increased agonistic potencies as compared to peptide I itself. A linear peptide, Ile-Ile-Trp-D-Trp-Leu-Asp, which presumably forms a similar cyclic conformation, was also shown to be a GnRH agonist. Intraperitoneal administration of Ac-Ile-Ile-Trp-D-Trp-Leu-Cys-OH ($K_i = 0.32\ \mu M$), one of the cyclic hexapeptides that we have synthesized, to rats induces secretion of luteinizing hormone (LH) with a potency which is only 1 order of magnitude less than that of GnRH itself. Moreover, plasma levels of LH remained elevated for a longer period of time following the administration of the cyclic hexapeptide. This novel class of GnRH agonists may prove useful in the development of new therapeutics.

Introduction

Gonadotropin-releasing hormone (GnRH) is a neurohormone that is secreted from the hypothalamus in a pulsatile pattern and regulates the reproductive system by controlling the secretion of the gonadotropic hormones, luteinizing hormone (LH), and follicle stimulating hormone (FSH) from the anterior pituitary.[1,2] On the basis of conformational energy calculations[3] and various physicochemical methods,[4] it was suggested that the bioactive conformation of GnRH include a type II′ β turn involving residues 5−8 {Tyr[5]-Gly[6]-Leu[7]-Arg[8]}.

The sequence of the peptide Ac-D-Trp-Leu-Asp-Ile-Ile-Trp (peptide I), which is an antagonist of the potent vasocostrictor endothelin (ET),[5] has no apparent similarity to that of GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). Nevertheless, we have previously demonstrated that peptide I can activate the pituitary GnRH receptor and induce LH secretion.[6] Peptide I, derived from the sequence of the C-terminal domain of ET, binds specifically to pituitary GnRH receptors with a moderate affinity ($K_i = 1.9\ \mu M$), whereas ET itself does not bind to the GnRH receptor.[6] We have also found that removal of the acetyl group of the peptide I results in a hexapeptide (D-Trp-Leu-Asp-Ile-Ile-Trp, peptide II) (Table 1) with similar properties with respect to the GnRH receptor, but with a diminished affinity toward the ET receptor.

In this study, we report structure−activity studies of peptide I with respect to the GnRH receptor. We have addressed both the issues of the conformation of the bioactive peptide and of the importance of specific residues for binding and activation of the GnRH receptor. Our studies resulted in partial characterization of the interactions of peptide I with the GnRH receptor, which may also add to the understanding of the interactions of GnRH with its receptor. Furthermore, we have designed stable analogues with improved potency, which may be utilized in the development of novel drugs that can stimulate the secretion of the gonadotropic hormones from the pituitary gland.

Results

Structural studies of peptide I using a combination of NMR and molecular dynamics suggest a cyclic conformation for this peptide in solution.[7] To test the possible relevance of such a conformation to GnRH receptor recognition, we have synthesized a head-to-tail cyclic analogue of the deacetylated peptide Ile-Ile-Trp-D-Trp-Leu-Asp (peptide III, Figure 1). This modification resulted in an undetectable ET receptor binding, in an increased GnRH binding affinity (Table 1), and in increased LH releasing activity (Figure 2). These results suggest that the apparent cyclic conformation of peptide I is recognized by the GnRH receptor. The assumption is schematically illustrated in Figure 1.

This notion is further strengthened by evaluation of peptide IV, Ile-Ile-Trp-D-Trp-Leu-Asp (see Figure 1). The sequence of this peptide was constructed since it may, potentially, form a semicyclic conformation similar to that suggested for peptide II. The GnRH receptor binding affinity (Table 1) and LH releasing activity

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Table 1. Hexapeptides Derived from Endothelin Antagonist: Mass Spectrometry and Binding to the GnRH and the Endothelin Receptors

peptide	sequence	MH^+ obsd (calcd)[a]	GnRH receptor K_i (μM)[b]	ET receptor K_i (μM)[c]
I	Ac-D-Trp-Leu-Asp-Ile-Ile-Trp	887.2 (887.0)	1.9 ± 0.5	0.12 ± 0.07
II	D-Trp-Leu-Asp-Ile-Ile-Trp	845.8 (845.5)	8 ± 1	55 ± 15
III	Ile-Ile-Trp-D-Trp-Leu-Asp	827.5 (827.0)	0.7 ± 0.1	NB
IV	Ile-Ile-Trp-D-Trp-Leu-Asp	845.5 (845.4)	0.7 ± 0.1	NB
V	hydantoin-D-Trp-Leu-Asp-Ile-Ile-Trp[d,e]	871.9 (871.7)	0.23 ± 0.04	6 ± 2
VI	Ac-Ile-Ile-Trp-D-Trp-Leu-Cys-OH[e]	872.9 (872.9)	0.32 ± 0.05	NB
VII	Ac-D-Trp-Leu-Arg-Ile-Ile-Trp	928.1 (927.9)	2.0 ± 0.6	NT

[a] Observed (obsd) and calculated (calcd) *m*/*z* values of MH^+ monoisotopes. In most cases, an additional peak, corresponding to MNa^+, was observed. Purities of the synthetic peptides were usually >98%, according to two analytical HPLC solvent systems which are detailed in the Experimental Section. [b,c] K_i = Inhibition constant for the displacement of specific binding of ^{125}I-[D-Lys^6]GnRH (K_d = 177 pM) (*b*) or of ^{125}I-ET-1 (K_d = 2 nM) (*c*) bound to rat pituitary membranes. Values are based on displacement curves obtained by incubating pituitary membranes with the respective tracer and increasing concentrations of the unlabeled peptides, as described in the Experimental Section. Nonspecific binding was determined in the presence of 1 μM of GnRH (*b*) or of ET-1 (*c*), and subtracted from the total binding for the calculation of specific binding. Results are mean ± SEM of two experiments carried out in triplicate. [d] Chemical name is [(*R*)-4(3-indolyl)methylimidazolidin-2,5-dione]-Leu-Asp-Ile-Ile-Trp. [e] For detailed structures, see Figure 2. NB = no significant binding at 10 μM (higher concentrations were not tested due to the presence of a high percentage of DMSO). NT = not tested.

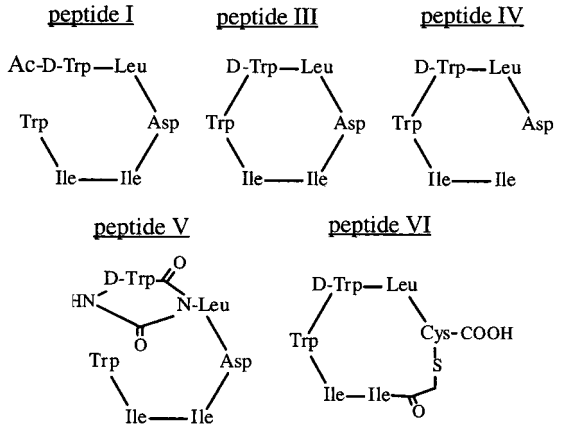

Figure 1. Schematic representation of the suggested constrained conformations of peptide I and its analogues.

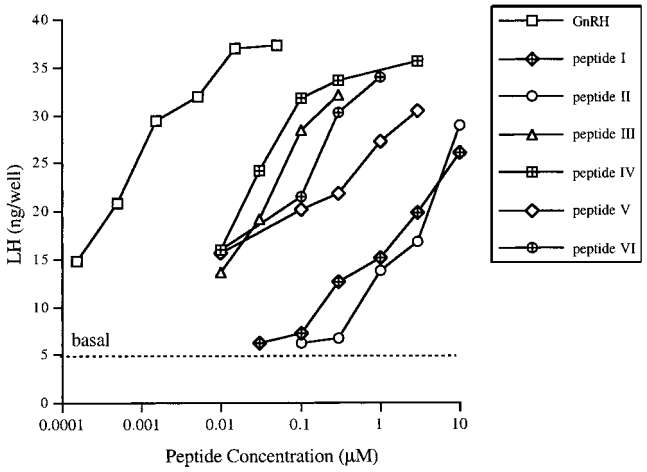

Figure 2. Induction of LH secretion from dispersed rat pituitary cells by increasing concentrations of GnRH and peptides I−VI. Primary pituitary cells were cultured for 48 h and subsequently incubated for 4 h at 37 °C with the examined peptides, following by determination of LH concentrations in the medium by using RIA. Results are the mean of LH concentration in two experiments (four wells/experimental group). The LH concentration in each well was determined using three different aliquots of medium. SEM values are omitted for clarity. There are no significant differences between the activities of peptides I and II, and also between the activities of peptides III, IV, and VI. All of the examined hexapeptides are significantly less potent than GnRH.

(Figure 2) of peptide IV are very similar to those of the cyclic peptide III. Thus, it seems that this peptide may indeed adopt a semicyclic conformation. The other four sequences which can theoretically form the same cyclic sequence {IWwLDI; WwLDII; LDIIWw; DIIWwL} [The sequences are written in the one-letter code for clarity. According to this code, the sequence of peptide II is wLDIIW, and that of peptide III is IIWwLD.] resulted in peptides which did not show significant GnRH receptor binding at 10 μM [Since all peptides are very hydrophobic, they were initially dissolved in dimethyl sulfoxide (DMSO) to obtain 1 mM stock solutions. Thus, in both binding and LH release bioassays, we were limited to 10 μM as the highest concentration which could be used without nonspecific DMSO interference (1% DMSO concentration). This limitation does not interfere with the distinction between ligands of interest, with significant activities at concentrations less than 10 μM, and other derivatives which possess much lower receptor affinities.], reflecting the specificity of the structures of peptide I and peptide IV, which are probably related to their enhanced tendency to be in a cyclic conformation (data not shown). The specificity of the sequence of peptide IV was further reflected in an Ala-scan of this peptide. None of the resulting peptides {AIWwLD; IAWwLD; IIAwLD; IIWaLD; IIWwAD; IIWwLA} showed more than 25% displacement of ^{125}I-[D-Lys^6]GnRH specific binding to rat pituitary membranes at 10 μM (the corresponding displacement by peptide IV is 90%).

The improved GnRH receptor binding affinity of peptide III over peptide I (Table 1) encouraged further studies of constrained analogues of peptide I. We found two distinct modifications which resulted in peptides V and VI, whose GnRH receptor binding affinities (Table 1) are about 10-fold higher than that of peptide I. While these affinities are significantly higher than those of peptides III and IV, only peptide VI was at least as potent as these peptides in the in vitro LH release assay, while peptide V was slightly less potent than peptides III, IV, and VI (Figure 2). The in vivo LH releasing activity of peptide VI following an intraperitoneal injection was investigated both in ovariectomized and in proestrous rats (Figure 3, A and B, respectively). In both models, 10 nmol of peptide VI has an activity similar

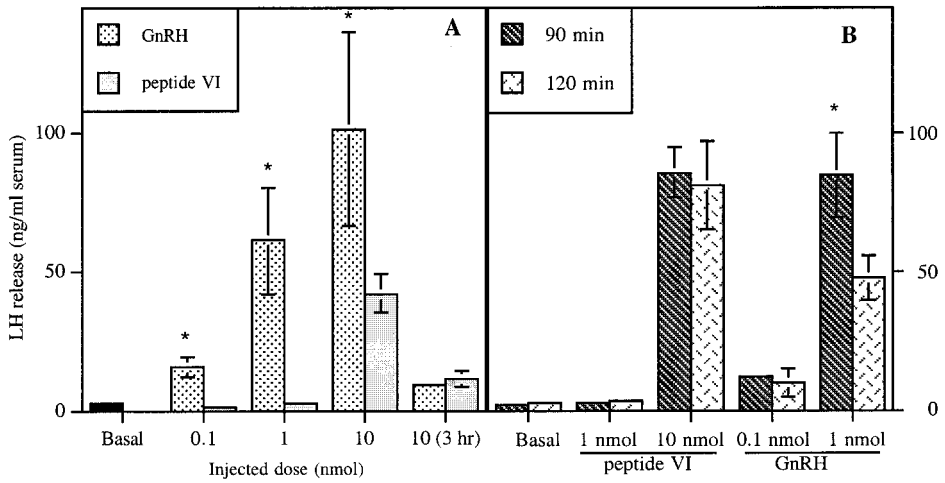

Figure 3. Induction of LH release in rats by intraperitoneal administration of GnRH or peptide VI. A: Ovariectomized rats were treated with estradiol benzoate (50 μg/rat) and progesterone (25 mg/rat) 3 days before peptide administration. Blood samples were taken from each rat at 45 min and, in the groups administered with 10 nmol of the peptides, also at 3 h after peptide injection. *Significantly higher LH level than that with the respective concentration of peptide VI ($p < 0.05$). B: Proestrous rats were intraperitoneally administered with GnRH or peptide VI at the indicated dose. Blood samples were taken from each rat at 90 and 120 min after peptide injection. *Significantly higher LH level than in the respective 120 min time point ($p < 0.05$). Results in both A and B are the mean ± SEM of LH concentrations in the serum of five animals/experimental group. The LH concentrations were determined by radioimmunoassay using three different dilutions of each serum sample.

to that of 1 nmol of GnRH. Thus, the in vivo potency of peptide VI is only about 10-fold lower than the respective activity of GnRH. However, the experiments in the two animal models show that peptide VI is long-acting as compared to GnRH. Figure 3A demonstrates that the LH levels in animals treated with 10 nmol of each of the peptides are similar after 3 h, although GnRH is significantly more active at the 45 time period; Figure 3B demonstrates that the LH levels of the GnRH-treated animals, but not of peptide VI-treated animals, are significantly decreased between the 90 and 120 time intervals. The high in vivo activity of peptide VI support our previous assumption regarding a constrained structure as the active conformation of peptide I and further demonstrate the potential usefulness of this peptide for the development of novel potent and long-acting GnRH analogues.

To test the selectivity of the developed GnRH agonists, we have studied their binding to pituitary ET receptors. The results (Table 1) suggest that the cyclic derivatives (peptides III, VI) and also peptide IV are highly selective ligands of the GnRH receptor. Peptide V, which is less constrained than the cyclic peptides, is less selective (Table 1). These results are compatible with studies showing that cyclization of ET antagonistic peptides resulted in the loss of affinity toward the ET receptor.[8]

To ensure that peptides III and IV are indeed activating the GnRH receptor, we have tested their activity in the absence or presence of antide, a powerful GnRH antagonist (Ac-β-[2-naphthyl]-D-Ala-D-*p*-chloro-Phe-β-[2-pyridyl]-D-Ala-Ser-N^{ϵ}-[nicotinoyl]-Lys-N^{ϵ}-[nicotinoyl]-D-Lys-Leu-N^{ϵ}-[isopropyl]-Lys-Pro-D-Ala-NH_2). Indeed, the LH releasing activities of peptides III and IV, as well as that of peptide I and GnRH, were diminished in the presence of the GnRH antagonist (Figure 4). Taken together, these observations demonstrate that the activity of peptide I is mediated directly by GnRH receptors.

To characterize the role of the two Trp residues of peptide IV (Ile-Ile-Trp-D-Trp-Leu-Asp) in receptor recognition and activation, we have synthesized analogues in which either of these residues was replaced by Phe and His. The results of the binding studies (Table 2) suggest a crucial role of the L-Trp residue of peptide IV in receptor recognition, while the D-Trp residue can be replaced by aromatic D-amino acids or by L-Trp without a loss of receptor recognition. The substitution of D-Trp by D-His results in an analogue with GnRH receptor affinity similar to that of peptide IV (Table 2). The LH releasing activities of the two peptides are also similar (data not shown).

Substitution of Asp in the sequence of peptide I by the structurally related though uncharged Asn residue

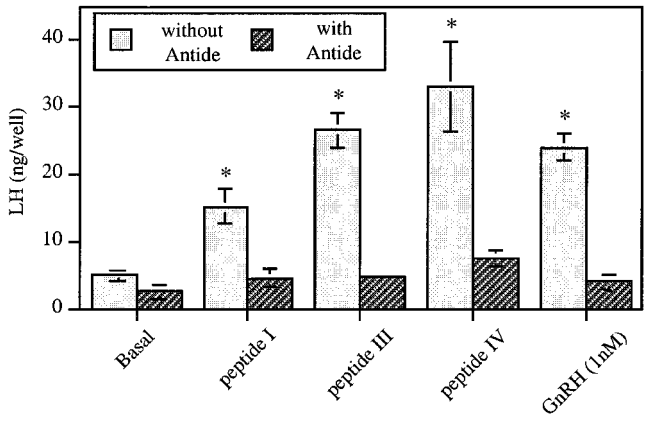

Figure 4. Effects of a GnRH antagonist (antide) on the induction of LH secretion from cultured rat pituitary cells by GnRH and peptides I, III, and IV. At 48 h after dispersion, the medium was changed, and the cells were incubated for 4 h at 37 °C with GnRH (1 nM) or with peptides I, III, and IV (1 μM each) in the absence (without Antide) or presence (with Antide) of antide, a GnRH antagonist (10 nM). The medium was collected, and LH concentration was determined by RIA. Results are the mean ± SEM of LH concentration of two experiments (four wells/experimental group). The LH concentration in each well was determined using three different aliquots of the medium. *LH release significantly higher ($p < 0.01$) than in the control group (basal) and the group of the respective peptide that was incubated with antide.

Table 2. Peptide IV and Its Analogues: Mass Spectrometry and Binding to the GnRH Receptor

sequence	MH^+ obsd (calcd)[a]	GnRH receptor K_i (μM)[b]
Ile-Ile-Trp-D-Trp-Leu-Asp (peptide IV)	845.5 (845.4)	0.7 ± 0.1
Ile-Ile-**His**-D-Trp-Leu-Asp	796.4 (796.4)	NB
Ile-Ile-**Phe**-D-Trp-Leu-Asp	806.6 (806.4)	NB
Ile-Ile-Trp-**D-His**-Leu-Asp	796.4 (796.0)	0.5 ± 0.2
Ile-Ile-Trp-**D-Phe**-Leu-Asp	806.5 (806.4)	8 ± 1
Ile-Ile-Trp-**D-Ala**-Leu-Asp	730.4 (730.1)	>10
Ile-Ile-Trp-**Trp**-Leu-Asp	845.5 (845.5)	4 ± 1

[a] Observed (obsd) and calculated (calcd) m/z values of MH^+ monoisotopes. In most cases, an additional peak, corresponding to MNa^+, was observed. Purities of the synthetic peptides were usually >98%, according to two analytical HPLC solvent systems which are detailed in the Experimental Section. [b] K_i = Inhibition constant for the displacement of specific binding of ^{125}I-[D-Lys^6]GnRH (K_d = 177 pM) bound to rat pituitary membranes. Values are based on displacement curves obtained by incubating pituitary membranes for 90 min at 4 °C with ^{125}I-[D-Lys^6]GnRH and increasing concentrations of the unlabeled peptides, as described in the Experimental Section. Nonspecific binding was determined in the presence of 1 μM of GnRH, and subtracted from the total binding for the calculation of specific binding. Results are mean ± SEM of two experiments carried out in triplicate. The variations from the sequence of peptide IV are indicated by **bold** letters. NB = no significant binding at 10 μM (higher concentrations were not tested due to the presence of a high percentage of DMSO).

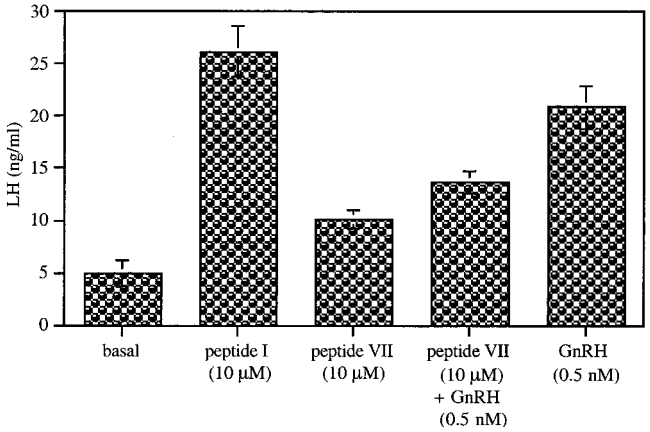

Figure 5. Induction of LH secretion from primary cultures of rat pituitary cells by GnRH, peptide I, and peptide VII. At 48 h after dispersion, the medium was changed, and the cells were incubated for 4 h at 37 °C with the examined peptides. The medium was collected, and LH concentration was determined by RIA. Results are the mean ± SEM of LH concentration of two experiments (four wells/experimental group). The LH concentration in each well was determined by radioimmunoassay using three different dilutions of each sample. LH release in the presence of each of the peptides is significantly higher ($p < 0.01$) than in the control group (basal). Induction of LH secretion by GnRH was significantly inhibited ($p < 0.01$) in the presence of peptide VII.

resulted in an analogue which did not show significant binding even at a concentration of 10 mM. Nevertheless, substitution of Asp by Arg resulted in peptide VII, which has GnRH receptor binding affinity similar to that of peptide I (Table 1). Thus, substitution of the negatively charged Asp residue by the positively charged Arg residue preserved the affinity for the GnRH receptor. Peptide VII, however, is a much weaker GnRH agonist with respect to peptide I, and it actually acts as a partial GnRH antagonist, since its incubation with GnRH resulted in a partial inhibition of GnRH action (Figure 5).

Discussion

The improved GnRH receptor affinity and the increased bioactivity of the cyclic peptide III over peptides I and II (Table 1, Figure 2) serve as strong evidence to suggest that a cyclic conformation of peptide I is recognized by the GnRH receptor. This assumption is also supported by our subsequent studies: the cyclic peptide VI possesses an even higher affinity to the GnRH receptor (about 6 times that of peptide I, Table 1). The increased GnRH receptor binding affinity (Table 1) and LH releasing activity (Figure 2) of peptide IV over peptide I and peptide II may be due to a higher tendency of peptide IV to adopt a cyclic conformation, which is the presumed active conformation. Moreover, peptides II and IV, but not the other four linear peptides which can theoretically form the same cyclic sequence {IWwLDI; WwLDII; LDIIWw; DIIWwL}, were found to bind to the GnRH receptor (the other peptides did not show significant GnRH receptor binding at 10 μM). The different affinities may be attributed to the nature of the terminal residues of each of these peptides: In peptide II {wLDIIW}, the terminal Trp and D-Trp residues may interact through $\pi-\pi$ interactions to facilitate a constrained conformation that is recognized by the GnRH receptor, although these bulky residues can also induce some sterical hindrance. In peptide IV {IIWwLD}, a constrained conformation can be triggered by two electrostatic interactions: between the terminal carboxyl and the terminal amine as well as between the terminal amine and the Asp carboxyl side chain. In all other four sequences {IWwLDI; WwLDII; LDIIWw; DIIWwL}, there is only one potential interaction between terminal residues: between the terminal carboxyl and the terminal amine. An alternative explanation for the different affinities may be based on the effect of the central region sequence of the peptide upon cyclization. However, no distinct differences were found between peptides II and IV as compared to the other four peptides in this respect.

The assumed receptor-bound cyclic conformation of peptides I and II may resemble the β turn motif of the middle part (residues 5−8) of the GnRH molecule.[9] Peptide III (Ile-Ile-Trp-D-Trp-Leu-Asp) resembles a cyclic hexapeptide GnRH analogue that was previously reported in a proceeding of a symposium.[10] This weak GnRH antagonist, Trp-Pro-Tyr-D-Trp-Leu-Arg, has certain similarities, i.e., hydrophobic Ile-Ile/Trp-Pro, followed by an aromatic Tyr/Trp and an identical D-Trp-Leu combination. The design of this weak antagonist was based on the middle domain (residues 5−8, Tyr^5-D-Trp^6-Leu^7-Arg^8) of the GnRH superagonist, (D-Trp^6)-GnRH. The most significant difference between the two molecules, however, is the Arg residue in the antagonist as compared to the Asp residue in the corresponding position of peptide III. We have demonstrated that replacing the respective Asp residue of the agonist peptide I by Arg results in peptide VII, which is a partial antagonist (Figure 5). Thus, it might be that an Arg residue in both Trp-Pro-Tyr-D-Trp-Leu-Arg and in peptide VII is detrimental for receptor activation. Appar-

ently, the respective Arg residue in GnRH itself does not interfere with receptor activation.

Our results may contribute to better understanding of the interactions of GnRH with its receptor, especially those interactions which are crucial for receptor activation. The residues considered to be most critical for GnRH agonist binding and activity, based on structure−activity studies, are pGlu[1], His[2], Trp[3], and the carboxy-terminal amide of Gly amide.[10,11] Among these residues only Trp is found in all of the hexapeptide GnRH agonists described above, and it may therefore account, at least partially, for their GnRH agonistic activity. Indeed, our studies suggest that the Trp (L-Trp) residue, rather than the D-Trp residue, is essential for receptor recognition. Nevertheless, a His residue substitution in this position results in equivalent GnRH receptor affinity (Table 2) and activity (data not shown). Thus, the Trp residues found in the hexapeptides may be equivalent to either His[2] or Trp[3] of GnRH.

Our structure−activity studies are very promising in two aspects related to potential drug development: higher GnRH receptor binding affinity was obtained by various modifications of the structure of peptide I (such as cyclization), and specificity toward the GnRH receptor was improved (Table 1). Thus, the application of peptide I as a lead compound toward the development of a new class of potent GnRH analogues resulted in more potent and specific analogues. Such analogues may have unique advantages over the currently known GnRH analogues by possessing better permeability through membranal barriers due to notable enhanced hydrophobicity and reduced size.[12] In addition, such analogues may exhibit higher metabolic stability toward enzymatic degradation due to the constrained structures (i.e., peptides III, VI). Indeed, the biological activity of peptide VI, in inducing LH secretion in vivo, was extended for a longer duration of time as compared to GnRH itself (Figure 3). The fact that the sequence of the analogues presented herein is distinct from that of GnRH may be used to treat patients which suffer from infertility due to insensitivity to GnRH, as a result of formation of anti-GnRH antibodies. Indeed, in a radioimmunoassay we did not observe any interaction between peptide I and a specific GnRH antibody. It might be also possible to convert these novel GnRH agonists into antagonists, as previously reported for GnRH analogue fragments of a comparable size.[13] A partial antagonist was already reported herein (peptide VII). Hexapeptide GnRH analogues may also serve as a good starting point for the development of non-peptide GnRH analogues.

Experimental Section

Materials. Unless otherwise stated, all chemicals and reagents were of analytical grade. Trifluoroacetic acid (TFA) for high performance liquid chromatography (HPLC) was obtained from Merck (Darmstadt, Germany). N^{α}-9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid derivatives and Wang, Rink-amide, and Cl-Trityl resins were purchased from Novabiochem (Laufelfingen, Switzerland). *N*,*N*′-disuccinimidyl carbonate, GnRH, antide, and endothelin-1 were acquired from Sigma (St. Louis, MO).

Peptide Synthesis. All peptides, other than the above, were prepared in our laboratory by solid-phase peptide synthesis, with an AMS-422 multiple peptide synthesizer (ABIMED, Langenfeld, GmbH) using Fmoc chemistry,[14] following the company's protocols.[15] All synthesized peptides were deprotected and cleaved from the resin using a solution of TFA:triethylsilane:anisole:water (17:1:1:1)[15] or reagent K solution (TFA:phenol:water:thioanisole:1,2-ethanedithiol; 33:2:2:2:1) for peptides containing Arg and Trp.[16] After 2 h at room temperature, the cleavage mixtures were filtered and the peptides were precipitated from the solution with peroxide-free dry ether at 0 °C. Precipitated peptides were washed with cold dry ether, dissolved in water or water/acetonitrile solution, and lyophilized. The crude peptides were subjected to semipreparative HPLC purification, performed on a Waters system composed of two model 510 pumps, model 680 automated gradient controller, and model 441 absorbance detector (Waters, Milford, MA). The column effluents were monitored by UV absorbance at 214/254 nm. HPLC prepacked columns employed (Merck, Darmstadt, Germany) were LichroCART 250−10 mm containing Lichrosorb RP-18 (7 μm) for semipreparative purifications and Lichrospher 100 RP-18, 250−4 mm (5 μm) and wide pore butyl (C4, 5 μm), 250−4.6 mm (J. T. Baker Inc., Phillipsburg, NJ) for analytical separations. Separations were achieved using gradients of acetonitrile in water containing 0.1% TFA. The homogeneity of the resulting peptides was tested by analytical HPLC using two solvent systems: (1) A 0.1% TFA in water, B 0.1% TFA in 75% acetonitrile; 20% B to 80% B at 1%/min; A 0.1% TFA in water, B 0.1% TFA in 75% 2-propanol; 0% B to 50% B at 1%/min, using Lichrosorb RP-18 column; (2) A gradient of 0% B to 100% B at 2.5%/min employing a wide pore butyl (C4) column. The purity of the peptides in both systems was usually higher than 98%, and always higher than 95%. Solutions containing purified peptides were lyophilized. Samples of each of the peptides were hydrolyzed (6 N HCl, 110 °C, 22 h, in a vacuum) and analyzed with a Dionex automatic amino acid analyzer. These results were also used for quantification of the peptide content in each preparation. The peptides were also analyzed by an LCQ mass spectrometry system (Finnigan, Bremen, Germany) using a nanospray ionization technique. The latter two analyses further confirmed the composition and purity of the products. Pure peptides were dissolved in dimethyl sulfoxide (DMSO) to obtain 1 mM concentration, and aliquots were kept frozen (−20 °C). The DMSO content in the preparations for bioassays, 1% or lower, was tested as a control and found to have no effect on GnRH receptor binding or LH release.

Ile-Ile-Trp-D-Trp-Leu-Asp (Peptide III). H-Ile-Ile-Trp-(Boc)-D-Trp-Leu-Asp(OtBu)-[Cl Trt resin] (25 μmol, synthesized as described above) was removed from the resin using a solution of dichloromethane (DCM):trifluoroethanol (TFE):AcOH (7:2:1) for 1 h and precipitated with ether. The crude peptide was lyophilized. Dry peptide (10 μmol) was dissolved in *N*,*N*′-dimethylformamide (DMF) (5 mL), and a solution of *N*,*N*′-dicyclohexylcarbodiimide/1-hydroxybenzotriazol (DCC/HOBT) (25 μmol each) in DMF was added. The solution was stirred overnight, afterwhich the reaction was completed according to analytic HPLC. The DMF was evaporated, and the crude peptide was deprotected by reagent K solution and further purified as detailed above. Yield: 2.8 mg (3.39 μmol; 13.5%, based on the initial amino acid-resin loading). Mass spectrometry: *m*/*z* 827.5 (MH^+), 849.5 (MNa^+) (calculated 827.0, 849.2, respectively).

[(*R*)-4(3-Indolyl)methylimidazolidin-2,5-dione]-Leu-Asp-Ile-Ile-Trp (Hydantoin-D-Trp-Leu-Asp-Ile-Ile-Trp-OH, Peptide V, See Figure 1 for Detailed Structure). D-Trp-Leu-Asp(OtBu)-Ile-Ile-Trp(Boc)-[Wang resin] (25 μmol, synthesized as described above) was reacted with *N*,*N*′-disuccinimidyl carbonate (6.4 mg, 50 μmol) in the presence of triethylamine (3.5 μL) in dimethylformamide (DMF) (0.8 mL). Solution pH was 8. The reaction was performed in a sintered plastic syringe which was shaken overnight in a mechanical shaker at room temperature. The solution was than removed, and the resin was washed (DMF × 3, DCM × 3). A negative ninhydrin test indicated the completion of the reaction. The peptide was cleaved from the resin using a cold reagent K solution and precipitated with ether. The crude peptide was

further purified as detailed above. Yield: 2.4 mg (2.75 μmol; 11%, based on the initial amino acid-resin loading). Mass spectrometry: *m/z* 871.7 (MH^+), 893.4 (MNa^+) (calculated 871.9, 893.9, respectively). Amino acid analysis: Asp:Ile:Leu ratios were 1.0:1.65:0.03 (22 h hydrolysis) and 1.0:1.96:0.26 (72 h hydrolysis). The incomplete regeneration of Leu on acid hydrolysis is due to its participation in a hydantoin ring which is much more resistant to acid hydrolysis than the amide bond. This interpretation was previously suggested for matching results of a very similar procedure.[17] Trp is completely destroyed during the acidic hydrolytic conditions.

Ac-Ile-Ile-Trp-D-Trp-Leu-Cys-OH (Peptide VI). Bromoacetyl-*N*-hydroxysuccinimide ester was prepared according to the procedure of Bernatowicz et al.[18] The pure compound was kept in a dry desiccator at 4 °C until use. Ile-Ile-Trp(Boc)-D-Trp-Leu-Cys(Trt)-[Cl Trt resin] (25 μmol, synthesized as described above) was reacted with bromoacetyl-*N*-hydroxysuccinimide (75 μmol) in the presence of triethylamine (5 μL) in *N*-methyl pyrrolidone (NMP) (0.5 mL). The reaction was performed in a sintered plastic syringe which was shaken overnight in a mechanical shaker. The solution was then removed, and the resin was washed (NMP × 1, DCM × 3). A negative ninhydrin test indicated the completion of the reaction. The resulting bromoacetylated peptide (BrAc-Ile-Ile-Trp-D-Trp-Leu-Cys) was removed from the resin using a cold solution of TFA:triethylsilane:anisole:dimethyl sulfide:water (16:1:1:1) and precipitated with ether. The crude peptide was dissolved in DMF (5 mL), and 0.1 mL of 0.5M $NaHCO_3$ was added, resulting in pH of 8. The solution was stirred overnight, afterwhich the cyclization reaction was completed according to analytical HPLC. The crude peptide was further purified as detailed above. Yield: 1.2 mg (1.37 μmol; 5.5%, based on the initial amino acid-resin loading). Mass spectrometry: *m/z* 872.9 (MH^+), 895.9 (MNa^+) (calculated 872.9, 895.9; respectively).

Animal Studies. Wistar-derived rats were obtained from the Weizmann Institute Animal Resource Center. Experiments were carried out in compliance with the regulations of the Weizmann Institute of Science.

Binding to the Pituitary GnRH Receptor. [D-Lys^6]-GnRH (synthesized in our laboratory) was iodinated by the chloramine T method,[19] and ^{125}I-[D-Lys^6]GnRH was purified (1700 μCi nmol^{-1}) by the analytic HPLC system as described above. The binding assay was conducted as previously described.[19] In brief, pituitary membranes (25 μg protein/tube, prepared from Wistar-derived proestrous rats) were incubated for 90 min at 4 °C with 50 000 cpm (23.5 pM) ^{125}I-[D-Lys^6]-GnRH, alone or in the presence of various concentrations of the unlabeled peptides, in a total volume of 0.5 mL of the assay buffer (10 mM Tris-HCl containing 0.1% bovine serum albumin [BSA]). The reaction was terminated by rapid filtration through Whatman GF/C filters. The filters were washed three times with cold assay buffer and counted in an Auto-Gamma Counting System (Packard, Meriden, CT). The experiments were performed in triplicate. Nonspecific binding was defined as the residual binding in the presence of excess of [D-Lys^6]-GnRH (1 μM). Specific binding was calculated by subtracting the nonspecific binding from the maximal binding, determined in the absence of any competing peptide. K_i values were calculated using the curve-fitting software program Enzfitter (Elsevier Biosoft, Cambridge, U.K.).

Binding of Peptides to Endothelin Receptors. ^{125}I-ET-1 was prepared as previously reported[20] and purified (1800 μCi nmol^{-1}) by an analytic HPLC system as described above. Preparation of membranes from both the pituitary gland and from brain tissues, as well as the binding assay, were performed in a procedure that is similar to that described for the GnRH receptor binding assay, except for several differences. Random cycling rats instead of proestrous rats were used; each tube contained 50 000 cpm (25 pM) ^{125}I-ET-1 and either membranes from brain (400 μg protein/tube) or from pituitary (50 μg protein/tube). The samples were incubated at 37 °C for 120 min. The nonspecific binding was determined in the presence of 1 μM ET-1.

LH Release from Cultured, Dispersed, Pituitary Cells. Pituitaries from 21-day-old Wistar-derived female rats were dispersed as previously described[21] and incubated in 96-well plates (50 000 cells/well) at 37 °C in M-199 medium containing 5% horse serum. After 48 h the cells were washed with M-199 medium containing 0.1% BSA and incubated for 4 h with M-199/0.1% BSA containing the desired concentrations of the various peptides (4 wells/experimental group). Three different aliquots from each medium sample were analyzed for LH concentration by radioimmunoassay (RIA)[22] using the kit kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) Rat Pituitary Program. Results are expressed in terms of the RP-3 reference preparation.

In Vivo LH Release. A total of 0.5 mL of 0.1 M PBS containing the desired concentration of tested peptide was intraperitoneally injected to rats during the morning of proestrous. The control group was injected with 0.5 mL of 0.1 M PBS. Each experimental group was comprised of five rats. Blood samples were withdrawn by cardiac puncture under light ether anesthesia at specified times after the drug administration. The serum samples were assayed for LH content as described above.

Statistical Analysis. Results are expressed as the mean ± SEM. Comparisons were made using one-way analysis of variance (Instat 2.01, GraphPad Software, CA). $P < 0.05$ was taken as significant.

Abbreviations. Abbreviations of common amino acids are in accordance with the recommendations of IUPAC. Additional abbreviations: antide, Ac-β-[2-naphthyl]-D-Ala-D-*p*-chloro-Phe-β-[2-pyridyl]-D-Ala-Ser-N^ϵ-[nicotinoyl]-Lys-N^ϵ-[nicotinoyl]-D-Lys-Leu-N^ϵ-[isopropyl]-Lys-Pro-D-Ala-NH_2; BSA, bovine serum albumin; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethyl sulfoxide; ET, endothelin; GnRH, gonadotropin-releasing hormone; HOBT, 1-hydroxybenzotriazol; HPLC, high performance liquid chromatography; LH, luteinizing hormone; PBS, phosphate buffered saline; pGlu, pyroglutamic acid; RIA, radioimmunoassay; SEM, standard error of the mean.

Supporting Information Available: Amino acid analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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