Position 2 and Position 2/Ala[15]-Substituted Analogs of Bovine Growth Hormone-Releasing Factor (bGRF) with Enhanced Metabolic Stability and Improved in Vivo Bioactivity[†,‡]

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In order to prepare GRF analogs with high activity in vivo, a strategy was undertaken to stabilize the peptide to dipeptidylpeptidase IV (DPP-IV), a protease found in plasma which inactivates native human and bovine GRF by cleavage of the Ala^2–Asp^3 bond. Replacement of the Ala^2 residue with Ser, Thr, or Gly in [Leu^{27}]bGRF(1–29)NH$_2$ resulted in peptides greatly stabilized against proteolysis in plasma, but having low inherent GH-releasing activity when tested in bovine pituitary cell cultures. Replacement of Gly^{15} with Ala^{15} was marginally effective in improving the in vitro bioactivity of this group of peptides. When tested for GH-hormone release in steers, however, the Thr^2,Ala^{15} analog was four times more potent than bGRF(1–44)NH$_2$. Eleven additional analogs from the [X^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ series were synthesized and evaluated for metabolic stability in bovine plasma and for GH releasing activity in steers in vivo and in rat pituitary cells in vitro. Two compounds, [Val^2,Ala^{15},Leu^{27}]dGRF(1–29)NH$_2$ and [Ile^2,Ala^{15},Leu^{27}]-bGRF(1–29)NH$_2$, had increased GH-releasing activity in steers over that of [Thr^2,Ala^{15},Leu^{27}]-bGRF(1–29)NH$_2$ and over a previously reported super-potent analog, [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-hGRF(1–29)NH$_2$.

Introduction

Growth hormone-releasing factor (GRF) is a 44-amino acid residue amidated peptide produced by the hypothalamus which stimulates the secretion of growth hormone (GH) from the pituitary.[3,4] It has been shown that the active core of biological activity resides in the N-terminal 27–29 residues.[5–9] Bovine and human GRF have Tyr-Ala at the N-terminus, and deletion of these two residues results in peptides essentially devoid of GH releasing activity.[7,8] In GRFs from most other species, position 2 is occupied by Ala (for review, see refs 9 and 10 and citations within).

GRF is rapidly inactivated by blood plasma both in vitro[11–14] and in vivo[11] via cleavage between the Ala^2–Asp^3 peptide bond. Dipeptidylpeptidase IV (DPP-IV) has been identified as the main enzyme responsible for this inactivation. Alanine at position 2 in GRF (position P$_1$ according to the nomenclature of Schechter and Berger[15]) is located at the scissile bond cleaved by DPP-IV and was chosen as the main target for modification. DPP-IV is reported to cleave N-terminal dipeptides from proteins or peptides having either an X-Ala, an X-Pro, or an X-Hyp as the N-terminal sequence with the preference for X being an aromatic amino acid (for review, see 16 and citations within). DPP-IV-like activity was found in blood from a number of species.[11–14]

In a search for GRF analogs with improved metabolic stability and enhanced potency in vivo, we have focused on substituting the native Ala^2 residue in bovine GRF with various natural amino acids. On the basis of the reported DPP-IV substrate specificity, we hypothesized that substituting position 2 in GRF with any amino acid other than Ala, Pro, or Hyp would result in more metabolically stable GRF analogs.[1,2] Our goal was to make a GRF analog resistant to DPP-IV-like activity found in blood while high potency for GH release was maintained.

[Leu^{27}]bGRF(1–29)NH$_2$(YADAIFTNSYRKVLGQ-LSARKLLQDILNR-NH$_2$) was chosen as the template for modification. A Leu^{27} substitution was used to replace easily oxidizable Met^{27} and to make GRF compatible with recombinant DNA synthesis and CNBr processing.[17] Initially, Gly, Ser, or Thr was chosen as substitutes at position 2 because of their perceived structural similarity to native Ala. Subsequently, a Gly^{15} → Ala^{15} modification was implemented, since such a replacement was reported to enhance GRF potency,[18–20] and 11 other DNA-coded amino acids were added to the series as substitutes at position 2.

We describe here the preparation of these compounds and an evaluation of their metabolic stability in bovine plasma in vitro, GH-releasing activity in bovine or rat primary anterior pituitary cell cultures in vitro, and GH-releasing activity in Holstein steers in vivo.

Table I. Stability of GRF Analogs in Bovine Plasma in Vitro

	plasma pool A		plasma pool B	
peptide	% intact peptide after 1 h[a]	$t_{1/2}$ (min)	k^a (min^{-1})	$t_{1/2}^b$ (min)
[Leu^{27}]bGRF(1–29)NH$_2$	39.8[g]	50	0.0171[f]	41 (+)
[Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$	56.2[h]	96	0.00797[d]	87 (+)
[Gly^2,Leu^{27}]bGRF(1–29)NH$_2$	73.3[c]	nd	0.00224[h]	309 (–)
[Gly^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$	89.3[e,f]	nd	0.00184[g]	377 (–)
[Ser^2,Leu^{27}]bGRF(1–29)NH$_2$	76.1[c,d]	nd	0.00266[c]	260 (+)
[Ser^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$	84.0[f]	nd	0.00193[g]	360 (+)
[Thr^2,Leu^{27}]bGRF(1–29)NH$_2$	75.6[c,d]	147	0.00207[g,h]	334 (±)
[Thr^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$	91.1[e]	297	0.00185[g]	375 (±)
[desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]hGRF(1–29)NH$_2$	80.0[d,f]	217	nd	nd (–)
bGRF(1–44)NH$_2$	nd	nd	0.0110[e]	63 (+)

[a] Data is presented as means from three experiments. [b] (3–29) fragment was either present (+) or absent (–) in the plasma incubation mixtures. For the two Thr^2-analogs, the (3–29)GRFs were not seen for the first 1 h of incubation but could be detected at later time points (±). The (3–29)GRFs were generated from the Ser^2 analogs faster than from the Thr^2 peptides, but their formation rates were much slower than those from the GRF peptides with the native Ala^2. nd = not determined. [c-h] Means within a column lacking a common superscript letter differ at $p < 0.05$.[40]

Results and Discussion

Stability of Ser^2, Gly^2, and Thr^2 Analogs in Bovine Plasma in Vitro. The stability of the analogs was examined in two experiments with two different pools of bovine plasma. The results are presented in Table I. GRF analogs with Ser^2, Gly^2, or Thr^2 substitutions were more stable than their Ala^2 counterparts, in both the [Leu^{27}]-bGRF(1–29)NH$_2$ and [Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ format. Compounds in the Ala^{15} series were more stable than their Gly^{15} counterparts. The determined half-life of [Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ was in the range of 66–96 min, depending on the plasma pool, and was about twice as long as the half-life of [Leu^{27}]bGRF(1–29)NH$_2$. The Ala^{15}-substituted counterparts of Ser^2-, Gly^2-, and Thr^2-modified GRFs also had longer half-lives, ranging from 360 to 375 min, as compared to their corresponding position 2 singly substituted analogs ($t_{1/2}$ range of 260–345 min). An additional extension of the half-lives of position 2/Ala^{15}-substituted analogs ranged from 12% to 38% over the half-lives of their singly-modified position 2 counterparts with Gly^2, Thr^2, or Ser^2 (Table I). On average, the singly position 2-modified analogs from this series were 3–8 times, and the corresponding position 2/Ala^{15}-GRFs were 6–9 times more stable than the parent peptide [Leu^{27}]bGRF(1–29)NH$_2$, depending on the bovine plasma pool.

[DesNH$_2$Tyr^1,D-Ala^2,Ala^{15}]hGRF(1–29)NH$_2$, a highly potent GRF along reported by Felix et al.,[19,20] did not produce any (3–29) fragment when incubated in bovine plasma (Table I). This observation is similar to the reported stability of this analog in human plasma in vitro[12] and confirms that the peptide is indeed an inactive substrate for plasma DPP-IV. Its half-life in bovine plasma in vitro was about 37% shorter than the half-life of [Thr^2,-Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ when these two analogs were incubated under the same conditions (Table I).

A (3–29) fragment was observed from cleavage of the Ala^2–Asp^3 peptide bond in [Leu^{27}]bGRF(1–29)NH$_2$, confirming the result reported previously.[13] A similar, but more slowly occurring, degradation pattern, leading to the formation of the (3–29) fragment, was observed for [Ala^{15},-Leu^{27}]bGRF(1–29)NH$_2$.

The identity of the metabolite from the Ala^{15} analog was verified by amino acid analysis of material isolated by HPLC from samples of [Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ incubated in plasma for 2 h (Table II). The Gly^2-modified analogs did not produce any detectable metabolites while the [Ser^2]- and, to a lesser extent, [Thr^2]GRFs produced

Table II. Amino Acid Composition of Metabolites Isolated from Analogs of [Leu^{27}]bGRF(1–29)NH$_2$ Incubated in Bovine Plasma[a]

amino acid	metabolites[b]		
	from Ser^2 analog	from Ser^2,Ala^{15} analog	GRF(3–29) from Ala^{15} analog
Asp	3.89 (4)	3.90 (4)	3.99 (4)
Thr	0.88 (1)	0.88 (1)	1.06 (1)
Ser	2.44 (2)	2.50 (2)	1.83 (2)
Glu	1.98 (2)	1.92 (2)	2.26 (2)
Gly	1.03 (1)	0.00 (0)	0.31 (0)
Ala	1.97 (2)	2.88 (3)	3.06 (3)
Val	1.28 (1)	1.34 (1)	1.17 (1)
Ile	1.79 (2)	1.84 (2)	1.88 (2)
Leu	4.94 (5)	4.98 (5)	5.03 (5)
Tyr	1.73 (1)	1.75 (1)	0.97 (1)
Phe	0.83 (1)	0.81 (1)	1.01 (1)
Lys	1.93 (2)	1.95 (2)	2.01 (2)
Arg	2.91 (3)	2.92 (3)	2.78 (3)

[a] Values in parentheses are the theoretical amino acid composition of the corresponding (3–29) fragments. [b] Plasma metabolites which eluted as a single peak from a Vydac C$_{18}$ column were a mixture of the (3–29) fragment, β-Asp^8-containing deamidation product of the full-length peptide, and some of the intact peptide present due to the incomplete HPLC peak separation. The material heterogeneity is reflected in the elevated Ser and Tyr content and was confirmed by Edman degradation and/or MS (see Results and Discussion for details).

a metabolite coeluting on HPLC with the appropriate (3–29) fragment. Although we did not isolate sufficient amounts of the metabolites derived from the Thr^2 or Thr^2,-Ala^{15} analogs for further characterization, in a separate experiment, we were able to show that indeed the (3–29) fragment was generated from these peptides when a purified porcine kidney-derived DPP-IV preparation was used in phosphate buffer at pH 7.4 (unpublished data from our lab).

Although the metabolites from the Ser^2 or Ser^2,Ala^{15} analogs coeluted with the authentic (3–29) fragment, the amino acid composition of the isolated metabolites from the overnight incubation mixtures of [Ser^2,Leu^{27}]bGRF-(1–29)NH$_2$ and [Ser^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ indicated heterogeneity. The Ser and Tyr contents were higher than expected for the (3–29) fragments and this indicated that a full length GRF might have been the major component in these mixtures (Table II). Ten cycles of Edman degradation performed on the metabolite fraction derived from [Ser^2,Leu^{27}]bGRF(1–29)NH$_2$ showed the presence of a minor component (ca. 5%) with the sequence of Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg, which corresponds to the N-terminus of [Leu^{27}]bGRF(3–29)NH$_2$.
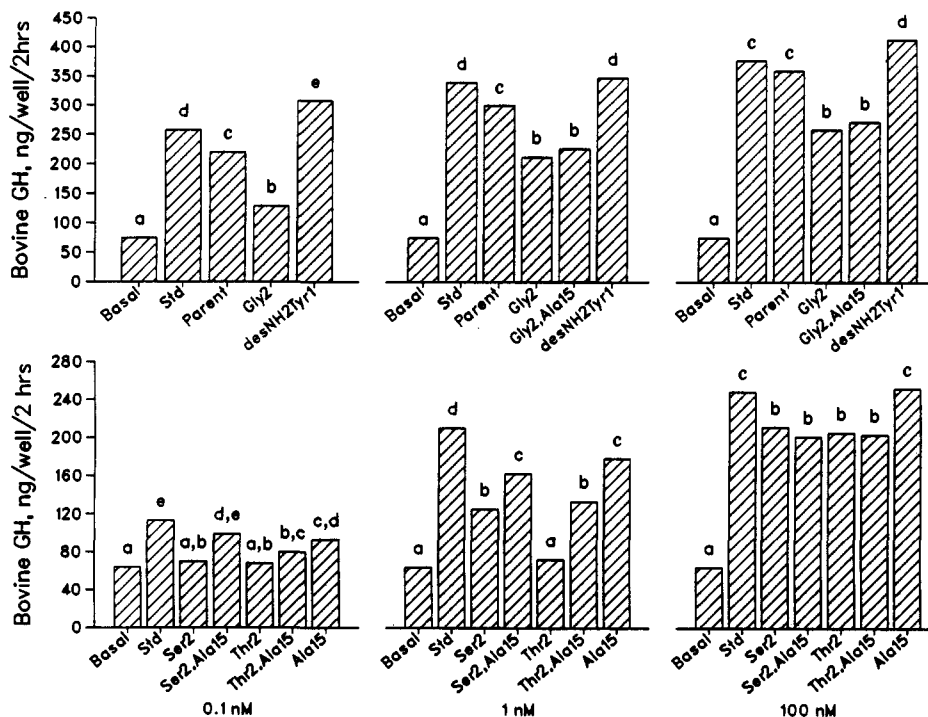
Figure 1. Effects of GRF analogs on GH release in bovine anterior pituitary cell cultures in vitro. Assay conditions were as described.[21] Std denotes bGRF(1-44)NH$_2$. a-d: Values within a cell isolation and a given GRF dose lacking a common letter differ at $p < 0.2$.[40]

Along with this GRF (3-29) fragment, sequencing analysis revealed ca. 20% of another minor component which had Asn at position 8 and was identified as intact [Ser^2,Leu^{27}]-bGRF(1-29)NH$_2$, possibly present due to the incomplete peak separation. The major component of the mixture was deduced to be [β-Asp^8,Leu^{27}]bGRF(1-29)NH$_2$. This was concluded on the basis of the greatly reduced sequencing yield after residue 7 (yield decreased to about one-third to one-quarter of the previously observed yield). The [β-Asp^8]GRF peptide, which copurified with [Leu^{27}]-bGRF(3-29)NH$_2$, was most likely generated from the intact peptide as a result of its pH-dependent deamidation during the prolonged incubation in plasma. This observation is in line with the report of Friedman et al.[21] on GRF deamidation in phosphate buffer at pH 7.4. The presence of [Leu^{27}]bGRF(3-29)NH$_2$ in the metabolite fraction shown by sequencing was also confirmed by liquid secondary ion mass spectrometry (data not shown).

In Vitro GH-Releasing Activity of Ser^2, Gly^2, and Thr^2 Analogs in Bovine Anterior Pituitary Cell Cultures. The GH-releasing potencies of Ser^2, Gly^2, or Thr^2 analogs of [Leu^{27}]bGRF(1-29)NH$_2$ were determined using assay conditions previously described.[21] Potencies relative to bGRF(1-44)NH$_2$ and [Leu^{27}]bGRF(1-29)NH$_2$ (respectively) were as follows: [Ser^2,Leu^{27}]bGRF(1-29)-NH$_2$, 14% and 24%; [Thr^2,Leu^{27}]bGRF(1-29)NH$_2$, 4.7% and (8.1%); and [Gly^2,Leu^{27}]bGRF(1-29)NH$_2$, 4.9% and (8.4%).

Subsequently, the activity of these analogs and their Ala^{15}-substituted counterparts was determined at three concentrations (0.1, 1, and 100 nM). The lowest dose (0.1 nM) represents approximately the EC$_{50}$ of native bGRF-(1-44)NH$_2$, while 1 and 100 nM have near-maximal and maximal efficacy, respectively. Compounds were allocated by two treatment groups and assayed with cells from a single cell isolation. The results are shown graphically in Figure 1. All analogs, at all doses, were significantly less active than bGRF(1-44)NH$_2$. The Gly^{15} → Ala^{15} modi-

fication in the Gly^2 analogs did not lead to increased activity. Also, these two peptides were least efficacious in the series, with the maximal GH release being about 70% of the maximal effect elicited by the bGRF(1-44)-NH$_2$ standard while all the others position 2-modified analogs from this series were 81%-85% as efficacious as the standard under the same experimental conditions (Figure 1). When tested at 0.1 and 1 nM, the analog with a single Thr^2 modification did not release significantly more GH than was observed for the nontreated control (basal). However, in combination with the Gly^{15} → Ala^{15} modification, the resulting analog released significantly more GH than the control at all doses tested. This trend was also observed in the Ser^2 and Ser^2,Ala^{15} analog pair.

Although the potency of the Ala^{15}, Ser^2,Ala^{15}, and Thr^2,-Ala^{15} compounds was not determined, the modest increase in activity of these compounds over their Gly^{15} counterparts in bovine pituitary culture appears to be less than the 5-fold increase in potency of [Ala^{15}]hGRF(1-29)NH$_2$ over hGRF(1-29)NH$_2$ and the 4-fold increase in potency over hGRF(1-44)NH$_2$[18-20] observed in rat pituitary cell cultures. This difference, however, may be species dependent (human GRF analog tested in rat anterior pituitary cell cultures vs bovine GRF analogs in bovine pituitary cell cultures).

[DesNH$_2$Tyr^1,D-Ala^2,Ala^{15}]hGRF(1-29)NH$_2$, a highly potent and DPP-IV-stabilized analog described by Felix et al.,[19,20] was only slightly more active (0.1 and 100 nM doses) than the native bovine hormone (Figure 1). This compound was reported to be 6-10-fold more potent than hGRF(1-29)NH$_2$ in rat anterior pituitary cell cultures in vitro[19,20] and this again may be a species-related difference as discussed for the Ala^{15}-substituted GRF analogs.

In Vivo GH-Releasing Activity of Ser^2, Gly^2, and Thr^2 Analogs in Steers (Study 1). The in vivo activity of the GRF analogs was measured using the meal-fed steer assay reported by Moseley.[22] This model standardizes

Table III. Serum GH Responses to Intravenous Injections of GRF Analogs with Substitutions at Positions 2 and 15 in the Meal-Fed Steer Assay in Vivo. Study 1[a]

peptide/treatment	animals responding [% (no.)]	GH peak height (ng/mL)	GH 0-2-h AUC (units)	relative activity[b] (%)
[Leu^{27}]bGRF(1-29)NH$_2$	89 (8/9)	38.3^{c,d}	1.8^c	106
[Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	78 (7/9)	72.0^{d,e}	2.0^c	118
[Gly^2,Leu^{27}]bGRF(1-29)NH$_2$	78 (7/9)	34.9^c	2.0^c	118
[Gly^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	89 (8/9)	41.0^{c,d}	2.3^c	135
[Ser^2,Leu^{27}]bGRF(1-29)NH$_2$	100 (9/9)	43.2^{c,d}	1.8^c	106
[Ser^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	89 (8/9)	57.4^{c,d}	2.4^{c,d}	141
[Thr^2,Leu^{27}]bGRF(1-29)NH$_2$	78 (7/9)	29.6^c	1.6^c	94
[Thr^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	89 (8/9)	92.9^e	3.3^d	195
bGRF(1-44)NH$_2$ (std)	78 (7/9)	38.4^{c,d}	1.7^c	100
SEM	11	13.2	0.3	
p value	0.85	0.01	0.02	

[a] Peptides were evaluated at the single intravenous dose of 0.01 nmol/kg bw injected 2 h after feeding. Their GH-releasing activities were determined by measuring serum GH levels as described by Moseley et al.[22] Data analysis includes only those animals which responded to the GRF injection. Graphical representation of these results was included in our earlier report.[1] [b] Relative GH-releasing activity was determined on the basis of the area under the 0-2-h GH response curve (GH AUC) for each analog as compared to the GH response to bGRF(1-44)NH$_2$ (assay standard) taken as 100%. [c-e] Values within a given column lacking a common superscript letter differ at $p < 0.05$.[40]

factors associated with the ingestion of feed which can modulate the release of GH from the pituitary.[22]

Bovine GRF(1-44)NH$_2$ and the parent [Leu^{27}]bGRF-(1-29)NH$_2$ were used as standards. A dose of 0.01 nmol/kg was selected (based on a minimally effective dose of native bGRF(1-44)NH$_2$, data not shown) in order to identify the more potent analogs. We rationalized that by using this low dose it would be possible to distinguish analogs more active than the native hormone though we might miss recognizing of less active GRFs. The area under the 0-2-h GH response curve was used to compute analogs bioactivities relative to that of bGRF(1-44)NH$_2$.

The serum GH response to the Gly^2-, Ser^2-, and Thr^2-substituted analogs and their Ala^{15}-modified counterparts is shown in Table III. Seventy-eight to 100% of the animals responded to injections. For the steers that responded, there was a significant main effect of treatment for GH peak height ($p = 0.01$), time to the GH peak ($p = 0.04$), and total area under the 0-2-h GH response curve ($p = 0.02$).

In study 1, all analogs were as active or, in the case of the Thr^2,Ala^{15} analog, more active than the parent peptide or native bGRF(1-44)NH$_2$ despite the lower inherent (in vitro) GH-releasing activity of the analogs. In vivo, [Thr^2,-Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$ released about twice as much GH as bGRF(1-44)NH$_2$ or [Leu^{27}]bGRF(1-29)NH$_2$.

Dose-Response Curves for [Thr^2,Ala^{15},Leu^{27}]bGRF-(1-29)NH$_2$ and bGRF(1-44)NH$_2$ in Vivo in Steers. Using multiple iv doses in the meal-fed steer assay,[22] the in vivo potency of [Thr^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$ relative to bGRF(1-44)NH$_2$ was determined. The dose-response curves were constructed from the 0-4-h area under the GH curves and were analyzed using regression lines in parallel-line analysis as shown in Figure 2. The potency of [Thr^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$ was determined to be 3.78, relative to bGRF(1-44)NH$_2$, and 95% confidence limits ranged from 2.1 to 6.98.

In Vivo Activity of Additional Analogs from the [X^2,Ala^{15}Leu^{27}]bGRF(1-29)NH$_2$ Series (Studies 2 and 3). Encouraged by the high activity of the Thr^2,Ala^{15} analog in vivo, we prepared additional X^2,Ala^{15} analogs of [Leu^{27}]bGRF(1-29)NH$_2$ for testing. Eleven GRF analogs from the [X^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$ series were assayed in the meal-fed steer assay at a dose of 0.01 nmol/kg administered as an iv bolus (study 2). bGRF(1-44)-NH$_2$ was the standard, and [desNH$_2$-Tyr^1,D-Ala^2,Ala^{15}]-
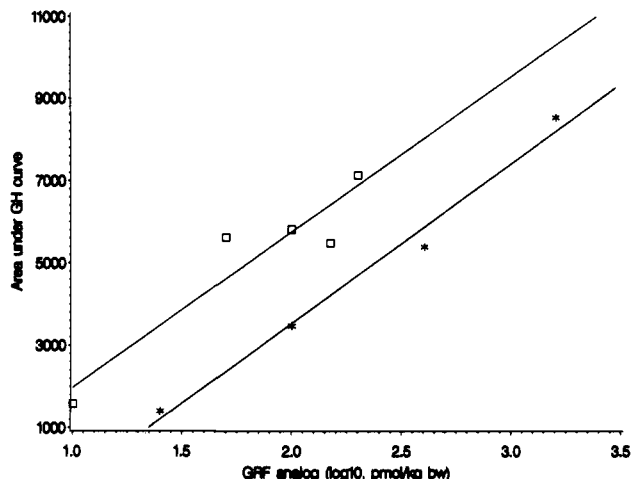

Figure 2. Dose-response GH curves after iv injection of [Thr^2,-Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$ (□) or bGRF(1-44)NH$_2$ (✳) in vivo in steers. The data are presented as the mean area under the GH response curve ($n = 10$ animals). Regression lines were used for parallel-line analysis.[36] The determined potency of [Thr^2,Ala^{15},-Leu^{27}]bGRF(1-29)NH$_2$ was 3.78 relative to that of bGRF(1-44)-NH$_2$ (potency = 1).

hGRF(1-29)NH$_2$ was included as a high-potency standard. The results of study 2 are presented in Table IV. Most of the analogs exhibited similar GH-releasing activity to that of bGRF(1-44)NH$_2$ ($p > 0.05$), ranging from 76% to 110% of the response obtained with this standard. Four compounds were significantly ($p < 0.05$) more active GH secretagogues than the native hormone. The analog-induced serum GH response relative to that of bGRF-(1-44)NH$_2$ is given: Ile^2, Ala^{15}, 180%; Val^{2}Ala^{15} 175%; and Thr^{2}Ala^{15} analog, 155%. The bioactivity of the high-potency standard, [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]hGRF-(1-29)NH$_2$, was 155%; however, in this assay, these three analogs and the high-potency standard were not significantly different ($p > 0.05$).

In order to better differentiate the in vivo activity of the compounds in the X^2,Ala^{15} series, a further study was run using the same meal-fed steer assay but higher doses of the test compounds and standards (study 3). The less active compounds from previous studies were tested at 0.1 nmol/kg while the more active compounds were tested at 0.02 nmol/kg. The results are presented in Table V. On the basis of the GH AUC response to peptide administration for the analogs tested at 0.1 nmol/kg, they can be

Table IV. Serum GH Responses to Intravenous Injections of GRF Analogs with Substitutions at Positions 2 and 15 in the Meal-Fed Steer Assay in Vivo. Study 2[a]

peptide/treatment	animals responding [% (no.)]	GH peak height[b] (ng/mL)	GH 0-2-h AUC (units)	relative activity[c] (%)
bGRF(1-44)NH2	80 (12/15)	32.7[d]	2.0[d]	100
[desNH2Tyr1,D-Ala2,Ala15]hGRF(1-29)NH2	93 (14/15)	72.0[e]	3.1[e]	155
[Thr2,Ala15,Leu27]bGRF(1-29)NH2	87 (13/15)	80.2[e]	3.1[e]	155
[Gln2,Ala15,Leu27]bGRF(1-29)NH2	67 (10/15)	42.6[d]	2.3[d]	115
[Asn2,Ala15,Leu27]bGRF(1-29)NH2	80 (12/15)	35.6[d]	2.1[d]	105
[Val2,Ala15,Leu27]bGRF(1-29)NH2	80 (12/15)	94.3[e]	3.5[e]	175
[Leu2,Ala15,Leu27]bGRF(1-29)NH2	93 (14/15)	42.7[d]	2.0[d]	100
[Ile2,Ala15,Leu27]bGRF(1-29)NH2	87 (13/15)	95.5[e]	3.6[e]	180
[Glu2,Ala15,Leu27]bGRF(1-29)NH2	80 (12/15)	29.1[d]	1.6[d]	80
[Phe2,Ala15,Leu27]bGRF(1-29)NH2	67 (10/15)	20.7[d]	1.6[d]	80
[Asp2,Ala15,Leu27]bGRF(1-29)NH2	73 (11/15)	29.1[d]	1.7[d]	85
[Arg2,Ala15,Leu27]bGRF(1-29)NH2	67 (10/15)	30.9[d]	1.7[d]	85
[Tyr2,Ala15,Leu27]bGRF(1-29)NH2	73 (11/15)	40.5[d]	2.0[d]	100
SEM	4	10.2	0.25	
p value	0.29	0.0001	0.0001	

[a] Peptides were evaluated at the single iv dose of 0.01 nmol/kg injected 2 h after feeding. Their GH-releasing activities were determined by measuring serum GH levels as described by Moseley et al.[22] Data analysis includes only those animals which responded to the GRF injection. Graphical representation of these results was included in our earlier report.[2] [b] The p value and superscripts are based on log transformed data. [c] Relative GH-releasing activity was determined on the basis of the area under the 0-2-h GH response curve (GH AUC) for each analog as compared to the GH response to bGRF(1-44)NH2 (assay standard) taken as 100%. [d,e] Values within a given column lacking a common superscript letter differ at $p < 0.05$.[40]

Table V. Serum GH Responses to Intravenous Injections of GRF Analogs with Substitutions at Positions 2 and 15 in the Meal-Fed Steer Assay in Vivo. Study 3[a]

treatment or compound name	dose (nmol/kg)	animals responding [% (no.)]	GH peak height (ng/mL)	GH 0-2-h AUC (units)	relative activity[b] (%)
sterile water	0	0 (0/17)	16.3[c]	0.95[c]	22
bGRF(1-44)NH2	0.1	100 (17/17)	133.4[i,j]	4.33[g,h]	100
[Gly2,Ala15,Leu27]bGRF(1-29)NH2	0.1	100 (17/17)	126.9[h-j]	6.37[i]	147
[Ser2,Ala115,Leu27]bGRF(1-29)NH2	0.1	100 (17/17)	123.8[h-j]	7.25[i]	167
[Gln2,Ala15,Leu27]bGRF(1-29)NH2	0.1	100 (17/17)	86.8[e,f]	3.36[e]	78
[Asn2,Ala15,Leu27]bGRF(1-29)NH2	0.1	100 (17/17)	103.9[f-h]	3.38[e,f]	78
[Lys2,Ala15,Leu27]bGRF(1-29)NH2	0.1	100 (17/17)	42.2[c,d]	1.64[c,d]	38
[Leu2,Ala15,Leu27]bGRF(1-29)NH2	0.1	100 (17/17)	143.1[j]	5.18[h]	120
[Glu2,Ala15,Leu27]bGRF(1-29)NH2	0.1	70 (12/17)	14.9[c]	0.92[c]	21
[Phe2,Ala15,Leu27]bGRF(1-29)NH2	0.1	100 (17/17)	56.0[d,e]	2.28[d]	53
[Asp2,Ala15,Leu27]bGRF(1-29)NH2	0.1	94 (16/17)	29.9[c,d]	1.64[c,d]	38
[Arg2,Ala15,Leu27]bGRF(1-29)NH2	0.1	88 (15/17)	23.7[c]	1.30[c]	30
[Tyr2,Ala15,Leu27]bGRF(1-29)NH2	0.1	88 (15/17)	24.2[c]	1.25[c]	29
[desNH2Tyr1,D-Ala2,Ala15]hGRF(1-29)NH2	0.02	100 (17/17)	82.9[f]	3.23[e]	75
[Thr2,Ala15,Leu27]bGRF(1-29)NH2	0.02	100 (17/17)	86.4[f,g]	3.11[e]	72
[Val2,Ala15,Leu27]bGRF(1-29)NH2	0.02	100 (17/17)	112.8[g-i]	4.22[f,g]	97
[Ile2,Ala15,Leu27]bGRF(1-29)NH2	0.02	100 (17/17)	127.5[h-j]	4.70[g,h]	109
SEM		4	10.2	0.3	
p value		0.0001	0.0001	0.0001	

[a] Peptides were evaluated at the single iv dose of 0.02 or 0.1 nmol/kg bw (as indicated) injected 2 h after feeding. Their GH-releasing activities were determined by measuring serum GH levels as described by Moseley et al.[22] Data analysis includes only those animals which responded to the GRF injection. [b] Relative GH releasing activity was determined based on the area under the 0-2-h GH response curve (GH AUC) for each analog as compared to the GH response to bGRF(1-44)NH2 (assay standard) taken as 100%. [c-j] Values within a given column lacking a common superscript letter differ at $p < 0.05$.[40]

assigned statistically ($p < 0.05$) to four groups: (i) analogs which induced serum GH not different from water-injected controls (Lys2,Ala15 Glu2,Ala15, Asp2,Ala15, Arg2,Ala15, and Tyr2,Ala15), (ii) more active than the water-injected controls but less active than bGRF(1-44)NH2 (activity range from 53% to 78%, $p < 0.05$) (Gln2,Ala15, Asn2,Ala15, and Phe2,Ala15); (iii) equally active to bGRF(1-44)NH2 ($p > 0.05$) (Leu2Ala15), and (iv) more active than bGRF(1-44)NH2 (activity 147% and 167%, respectively, $p < 0.05$) (Gly2,Ala15 and Ser2,Ala15).

For the analogs assayed at 0.02 nmol/kg, [Thr2,-Ala15,Leu27]bGRF(1-29)NH2 was as active as [desNH2-Tyr1,D-Ala2,Ala15]hGRF(1-29)NH2. Both compounds released significantly ($p < 0.05$) less GH (72%-75%) than bGRF(1-44)NH2 administered at a dose 5 times higher (0.1 nmol/kg). The Ile2,Ala15 and Val2,Ala15 analogs, the most active compounds in this series, were about 25%-28%

more active than [desNH2Tyr1,D-Ala2,Ala15]hGRF(1-29)-NH2. These two highly potent analogs were estimated to be about 5 times more potent than the native hormone bGRF(1-44)NH2 since a 5-fold lower dose of the analogs stimulated the same GH response as bGRF-(1-44)NH2 (Table V). Although a full dose-response curve was not run for these analogs, the estimate was based on the finding that 0.1 nmol/kg dose of bGRF(1-44)NH2 is on a linear portion of the dose-response curve as shown previously, and the assumption that the 0.02 nmol/kg dose of the [Val2,Ala15] or [Ile2,Ala15]GRFs is on a linear portion of their respective dose-response curves as was demonstrated previously for [Thr2,Ala15,Leu27]bGRF(1-29)NH2 under similar assay conditions.

The higher doses used in this study confirmed the high GH-releasing activity of the Ile2-, Val2-, and Thr2-modified analogs from the series [X2,Ala15,Leu27]bGRF(1-29)NH2

Table VI. Effect of bGRF Analogs on GH Release in Rat Anterior Pituitary Cell Cultures in Vitro[a]

analog	rat GH released[a] at analog concn listed below			
	0.005 nM	0.05 nM	0.05 nM	5 nM
Assay 1				
baseline (no analog added)	1431[b,c]	1431[b,c]	1431[b]	
[Leu^{27}]bGRF(1-29)NH$_2$	1921[e]	4735[f]	13605[e]	
[Thr^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	1486[c]	2276[d]	7801[d]	
[Val^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	1728[d]	4249[e]	13307[d,e]	
[Leu^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	1499[e]	1603[b,c]	2715[c]	
[Ile^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	1816[d,e]	49551[f]	12705[d]	
[Glu^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	1334[b,c]	1279[b]	1433[b]	
[Phe^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	1291[b]	1475[b,c]	1683[b]	
SEM	64.3	102.2	219.5	
p value	0.0001	0.0001	0.0001	
Assay 2				
baseline (no analog added)		1147[b,c]	1147b[b]	1147[b]
[Leu^{27}]bGRF(1-29)NH$_2$		4175[d]	12377[f]	17492[h]
[Gln^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$		1260[b,c]	1654[c,d]	6782[e]
[Asn^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$		1204[b,c]	1954[d,e]	8065[f]
[Lys^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$		1150[b,c]	1141[b]	1773[c]
[Asp^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$		1057[b]	2075[e]	8886[g]
[Arg^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$		1308[c]	1182[b]	1302[b]
[Tyr^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$		1166[b,c]	1495[b,c]	5267[d]
SEM		81.9	132.7	187.4
p value		0.0001	0.0001	0.0001

[a] The amount of rat GH released in the pituitary cell cultures was measured by radioimmunoassay and is expressed as ng per 2 × 10⁵ cells/4 h (n = 4). Assay conditions were as described.[23,24] A graphical representation of these results is included in our earlier report.[2] [b-h] Within a given assay, and within a given column, means lacking a common superscript letter differ at p < 0.05.[40]

and that of [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]hGRF(1-29)NH$_2$. Specifically, these doses, along with a higher number of animal treatments, allowed discrimination between the two most active compound in the series, Ile^2,Ala^{15} and Val^2,Ala^{15} analogs, from the slightly less active analogs [Thr^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$ and [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]hGRF(1-29)NH$_2$.

In Vitro Bioactivity of Analogs from the [X^2,Ala^{15},-Leu^{27}]bGRF(1-29)NH$_2$ Series in Rat Anterior Pituitary Cell Assay. In order to better understand some of the factors which contribute to the in vivo activity of each of the new X^2,Ala^{15} analogs, we examined them for their in vitro inherent GH-releasing activity using a rat cultured anterior pituitary cell assay.[23,24] Using pooled anterior pituitary cells from a single set of animals, the GRF analogs were divided into two assay groups (largely on the basis of their in vivo activity) and each group was assayed at three different doses for GH release (assay 1, 0.005, 0.05, and 0.5 nM; assay 2, 0.05, 0.5, and 5 nM). For the assay standard, [Leu^{27}]bGRF(1-29)NH$_2$, a full dose–response curve was run in each assay (five doses ranging from 0.005 to 50 nM). The EC$_{50}$ determined was 0.167 nM (0.143–0.190 nM, 95% confidence limit) in assay 1 and 0.234 nM (0.197–0.27 nM, 95% CL) in assay 2. The results are presented in Table VI. None of the position 2/Ala^{15}-modified analogs from the present series was more active than the parent peptide. The GH-releasing activities of [Val^2,Ala^{15}] and [Ile^2,Ala^{15}]GRFs were similar to the parent compound while the Thr^2,Ala^{15} analog was significantly less active at all three doses tested (p < 0.0001). The Leu^2,Ala^{15} analog, despite its structural similarity to the Val^2,Ala^{15} and Ile^2,Ala^{15} analogs, had significantly lower activity. The Leu^2,Ala^{15} analogs was also less active than the [Thr^2,Ala^{15}]GRF at all the doses tested, indicating that at position 2, amino acids with β-carbon branching may fit the GRF receptor better than γ-branched Leu. The lower activity of Leu analogs relative to Val or Ile analogs has also been observed when these substitutions were made at position 19.[25] The remaining analogs were

Table VII. Stability of GRF Analogs in Bovine Plasma in Vitro[h]

compound	plasma pool C: % intact peptide after 1 h	plasma pool D	
		k × 10⁴ (min⁻¹)	t$_{1/2}$ (min)
[Leu^{27}]bGRF(1-29)NH$_2$	46.8 ± 4.1[a] (+)	179 ± 5.6	39
[Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	54.1 ± 1.5[a,b] (+)	100 ± 4.4	69
[Thr^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	91.1 ± 3.1[i] (−)	27 ± 3.6	257
[Val^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	83.4 ± 5.4[d-f] (−)	16 ± 1.2	433
[Ile^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	80.3 ± 17.5[c-f] (−)	11 ± 0.6	630
[Leu^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	86.3 ± 4.0[e-f] (−)	nd	nd
[Glu^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	83.9 ± 1.5[d-f] (−)	nd	nd
[Phe^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	88.1 ± 3.41[f] (−)	nd	nd
[Gln^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	71.0 ± 4.2[c-d] (−)	nd	nd
[Asn^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	71.7 ± 8.0[c-d] (−)	nd	nd
[Lys^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	82.7 ± 5.4[d-f]	nd	nd
[Asp^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	89.4 ± 3.2[f] (−)	nd	nd
[Arg^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	88.0 ± 10.5[r] (−)	nd	nd
[Tyr^2,Ala^{15},Leu^{27}]bGRF(1-29)NH$_2$	107.0 ± 2.18[g] (−)	nd	nd

[a-g] Within a given assay, means lacking a common superscript letter differ at p < 0.05.[40] nd = not determined. [h] C and D denote two different plasma pools; each experiment was done in triplicate. [i] (+)/(−) Indicates that the (3–29) fragment was either generated (+) or not generated (−) in plasma.

also significantly less active (p < 0.0001) than [Leu^{27}]-bGRF(1-29)NH$_2$ even when tested at a high dose of 0.5 nM (assay 1) or 5 nM (assay 2). The Lys^2,Ala^{15}, Arg^2,-Ala^{15}, Glu^2,Ala^{15}, and Phe^2,Ala^{15} analogs were not significantly different from the basal GH levels (p > 0.05) over the entire concentration range tested (Table VI).

Stability of Analogs from the [X^2,Ala^{15},Leu^{27}]bGRF-(1-29)NH$_2$ Series in Bovine Plasma in Vitro. In order to characterize the stability of the newer X^2,Ala^{15} analogs to plasma enzymes, first, the stability index (percent of compound remaining after a 1-h incubation) was determined in bovine plasma. All of the X^2 analogs had a higher stability index (71%–100% range) than the parent GRF peptide (47%) and its Ala^{15} counterpart (54%) (Table VII). Stabilization against plasma DPP-IV was indicated by the absence of (3–29) fragments in the 1-h incubation mixtures of the analogs in bovine plasma. No other metabolites were identifiable under the HPLC conditions

used in the study, and no new cleavage sites specific for other plasma enzymes appeared to have been generated as a consequence of the position 2 substitutions.

In addition to the DPP-IV mediated cleavage in hGRF, Frohman et al.[11,12] also reported on the hydrolysis of hGRF by trypsin-like enzymes present in human plasma. As we reported previously,[13] no peaks corresponding to synthetic trypsin cleavage products were observed from incubation of [Leu[27]]bGRF(1-29)NH$_2$ in bovine plasma, nor were they observed in this study from [Leu[27]]bGRF(1-29)NH$_2$ or its analogs. It is unlikely that the substitutions at positions 27 (Met[27] → Leu) and 28 (Ser[28] → Asn) in our GRF analogs, which account for the differences between analogs of bGRF(1-29)NH$_2$ as compared to hGRF(1-29)-NH$_2$, are responsible for the differences between our results and Frohman's. We, therefore, attribute the observed differences to a different enzymatic activity spectrum of human and bovine plasma and the lack of apparent trypsin-like activity observed in the latter. In this sense, bovine plasma may be more like porcine plasma since Su et al.[14] did not report any trypsin-like cleavages in hGRF(1-29)-NH$_2$ or [Ala[15]]hGRF(1-29)NH$_2$ when these GRFs were incubated in porcine plasma in vitro.

For the Thr[2],Ala[15], Val[2],Ala[15], and Ile[2],Ala[15] analogs, the first-order disappearance constants and plasma half-lives were determined in bovine plasma in vitro and compared with those of [Leu[27]]bGRF(1-29)NH$_2$ and [Ala[15],Leu[27]]bGRF(1-29)NH$_2$. The results are presented in Table VII. The half-life of the Thr[2],Ala[15] analog (257 min) was 6.6 times longer than that of the parent peptide and 3.7 times longer than that of the Ala[15] analog. The half-lives of the Val[2],Ala[15] and Ile[2],Ala[15] peptides were 436 and 630 min, respectively, 11.2 and 16.2 times longer than that of [Leu[27]]bGRF(1-29)NH$_2$ and 6.3 and 9.1 times longer than the $t_{1/2}$ of the Ala[15] analog. The greater plasma stability of the [Val[2],Ala[15]] and [Ile[2],Ala[15]]GRFs seem to be due to the fact that these analogs, in contrast to their Thr[2] counterpart, were not cleaved by plasma DPP-IV. In both cases, neither (3-29) fragments nor any other major metabolites were detected under the HPLC conditions used in the study over the entire 25-h incubation period. The increased stability of the ILe[2] analog over the Val[2] analog may also be related to its increased hydrophobicity. This characteristic may serve to protect the analog from other plasma proteases, perhaps by hydrophobic interaction with serum components.

General Comments. Although short chromogenic substrates with Gly at P$_1$ had been previously reported to be resistant to hydrolysis by DPP-IV,[26] at the time of this study, no information on metabolic stability of GRF analogs substituted at position 2 with Gly had been available. Tou et al.[27] described a GRF analog modified by Ser[2] replacing Ala[2] as one of eight modifications made to hGRF(1-29)NH$_2$. This compound showed some improvement in activity in vivo in sheep, as compared to the native hormone and other analogs in the same series. Although this compound may have been protected against DPP-IV, due to the multiple substitutions, it was not clear how Ser[2] contributed to the overall bioactivity and/or stability of the analog. Recently, Bongers[28] reported that [Ser[2],Ala[15]]hGRF(1-29)NH$_2$ was cleaved at the 2-3 bond by human placental DPP-IV. In addition to our preliminary reports[1,2] showing improved stability of X[2]-modified GRFs, two additional reports[28,29] showed the improved stability to placental DPP-IV of a further series X[2]

(including Ile[2] and Val[2]) analogs of GRF. These observations are in a good agreement with our findings with regard to the stability of the analogous bovine GRF analogs against bovine plasma DPP-IV. Recently, during the course of this study, the sequence of mouse GRF was reported showing Val at the 2 position.[30,31]

The substitution of Ala for Gly at position 15 increases the plasma stability of all the analogs where this comparison could be made. The Ala[15] substitution alone in [Leu[27]]bGRF(1-29)NH$_2$ yielded an analog about twice as stable in bovine plasma as the parent compound, with a concurrent slower formation of the (3-29) fragment. The partial protection of the 2-3 bond in [Ala[15],Leu[27]]bGRF-(1-29)NH$_2$ was surprising since this analog was not modified at the N-terminus. A similar observation was made by Su et al.[14] with regard to [Ala[15]]hGRF(1-29)-NH$_2$ incubated with porcine plasma in vitro. The effect of the Gly[15] to Ala[15] modification on plasma stability is not well-understood. However it may be indirectly related to the increase in helicity[18] and hydrophobicity imparted by this modification. Both helicity and hydrophobicity were correlated with plasma stability in a series of GRF analogs modified at position 19.[25]

The performance of GRF analogs in vivo is a function of both metabolic stability and inherent GH-releasing activity. On the basis of the results presented here it appears that, for a metabolically stabilized GRF analog to be active in vivo, its inherent activity at the receptor must be above a certain threshold value. In that light, despite effective protection against DPP-IV, the analogs either inactive (Glu[2],Ala[15], Arg[2],Ala[15], or Lys[2],Ala[15]) or only weakly active (Asp[2],Ala[15] or Tyr[2],Ala[15]) in the rat pituitary cell cultures in vitro also weakly released GH in vivo in steers, as defined by percent of animals responding to treatment, but with the GH levels generated by treatment not different from the water-injected controls. In this case stabilization against DPP-IV, which possibly led to a longer life span of the intact peptides in the circulation, did not offset deleterious effects of their low inherent GH-releasing activity. On the other hand, moderate in vitro bioactivities of some GRF analogs (e.g. Gly[2], Gly[2],Ala[15], Ser[2], Ser[2],Ala[15], Thr[2], Leu[2],Ala[15], or Thr[2],Ala[15], combined with their enhanced plasma stability, produced serum GH response in steers either comparable to or higher than the treatment with the native bGRF(1-44)NH$_2$. Here, the improvement in metabolic stability was able to override low inherent potency and result in full or even enhanced performance of the GRF analogs in vivo. A similar observation applies to the Ile[2],Ala[15] and Val[2],Ala[15] analogs which were comparable to the unsubstituted parent peptide in their in vitro bioactivity but turned out to be 5 times as potent as bGRF(1-44)NH$_2$ in vivo and were identified as the most potent analogs from this series.

[DesNH$_2$Tyr[1],D-Ala[2],Ala[15]]hGRF(1-29)NH$_2$ has been reported by others to have 10-15 times the in vivo potency of hGRF(1-44)NH$_2$ in inducing blood GH[32] and to be more potent than hGRF(1-29)NH$_2$ in stimulating GH release and milk production of diary cows.[33] The lower estimated GH-releasing potency of this analog in our in vivo model in steers (only 4 times higher than that of bGRF(1-44)-NH$_2$) may be due to a different experimental design, different animals (dairy cows versus steers) as well as different standards used in these in vivo experiments. The lower stability in bovine plasma of [Thr[2],Ala[15],-Leu[27]]bGRF(1-29)NH$_2$ and [desNH$_2$Tyr[1],D-Ala[2],Ala[15]]-

hGRF(1–29)NH$_2$, determined in our study, may partially explain the slightly lower GH responses to these two analogs in vivo in steers as compared to the more metabolically stable [Val^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ and [Ile^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$. These results once more emphasize the significant contributions of both improved metabolic stability and inherent potency in increasing the GH-releasing activity observed in vivo.

It is also worthwhile to comment on the dose-dependent GH-releasing activity in vivo of the Ser^2,Ala^{15} analog. This peptide was not different from bGRF(1–44)NH$_2$ when tested in steers at a single iv dose of 0.01 nmol/kg, but a dose 10 times higher resulted in a 67% greater serum GH response in vivo than was induced by the native hormone. The DPP-IV-related degradation of [Ser^2,Ala^{15},Leu^{27}]-bGRF(1–29)NH$_2$, which was observed in bovine plasma in vitro, could have been accelerated under the in vivo conditions, where overall organ and tissue DPP-IV activity may be much higher than the plasma DPP-IV levels. The DPP-IV activity of the bovine plasma might represent only a small fraction of the overall organ and tissue DPP-IV activity, as has been reported for the rat system.[34] It is possible that a higher dose of [Ser^2,Ala^{15},Leu^{27}]bGRF-(1–29)NH$_2$ used in vivo led to a greater fraction of the intact peptide remaining in serum above a threshold level needed to increase serum GH. Therefore, it may be concluded that even a partial protection of GRF against DPP-IV cleavage, like in the Ser^2,Ala^{1}15 analog, is sufficient to improve peptide performance in vivo.

Summary

The present study on analogs of [Leu^{27}]bGRF(1–29)-NH$_2$, designed for improved metabolic stability against DPP-IV, resulted in three position 2-modified (Ser^2, Gly^2, and Thr^2) and 14 position 2/Ala^{15}-substituted GRF analogs which were either partially (Ser^2,Ser^2,Ala^{15},Thr^2, and Thr^2,-Ala^{15}) or fully (all remaining analogs) resistant to bovine plasma DPP-IV. The study led to the identification of three novel highly GRF analogs, [Thr^2,Ala^{15},Leu^{27}]bGRF-(1–29)NH$_2$, [Val^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, and [Ile^2,-Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, which were, respectively, 4, 5, and 5 times as potent as bGRF(1–44)NH$_2$ in releasing serum GH in vivo in Holstein steers. In vitro (rat system) the Thr^2,Ala^{15} analog was less active while the Val^2,Ala^{15} and Ile^2,Ala^{15} peptides were comparable to [Leu^{27}]bGRF-(1–29)NH$_2$. None of the remaining analogs from this series, was more active than the native sequence in releasing GH in vitro. The results presented here show that both inherent potency and metabolic stability are important for peptide performance in vivo, however, specific examples seem to highlight a greater contribution coming from improved metabolic stability which may override low inherent potency and result in full or even enhanced performance of the GRF analogs in vivo.

Experimental Section

Bovine GRF(1–44)NH$_2$ was purchased from Bachem, Inc. Other peptides were synthesized by the Merrifield solid-phase method[35] and purified using procedures described previously[13] except for the ion-exchange purification step, which was omitted. The synthetic peptides were of greater than 98% purity as judged by HPLC. The following are analytical HPLC conditions: column, a 0.46 × 15 cm Vydac C$_{18}$ (218TP5415); Perkin-Elmer 2600 system; gradient, 30%–50% acetonitrile/water, each containing 0.1% TFA over 30 min; detection at 215 nm. An outline of HPLC conditions of samples for plasma stability follows: column, 0.45 × 5 cm Vydac C$_{18}$ (218TP5405); flow rate, 2 mL/

min; detection at 215 nm, Hewlett-Packard Model 1090M system with various 0.1% aqueous TFA/acetonitrile gradients as previously described.[13] For amino acid analysis, peptides were hydrolyzed under vacuum by a vapor-phase method in a Pico-Tag Work Station (Waters Associates, Milford, MA) using constant-boiling HCl (Pierce) in the presence of phenol as scavenger at 110 °C for 24 h. Hydrolysates were analyzed on a Beckman amino acid analyzer, Model 7300. Conditions for mass spectral (MS) analysis (synthetic peptides characterization) follow: plasma desorption Cf-252 time-of flight mass spectrometer (Bio-Ion Model 20, Applied Biosystem); samples (3 nmol in 5 μL of 50% EtOH/0.1% TFA) applied to nitrocellulose-coated aluminized mylar foils and bombarded at an approximate rate of 1700 fission fragments per second; acceleration voltage, 15 kV; data acquisition time, 60 min. Conditions for MS (plasma metabolite characterization) follow: liquid secondary ion mass spectrometry (LSIMS), performed on a VG 70-SE double focusing mass spectrometer equipped with a cesium ion gun; Cs ions at 35 kV; a 1-μL mixture of *m*-nitrobenzyl alcohol/acetic acid (1:1, v/v) was used as the liquid matrix. All the peptides used in the study, including commercial bGRF(1–44)NH$_2$, had acceptable amino acid composition and the correct molecular weight values as determined by PDMS (data included as in supplementary materials).

Stability of GRF Analogs in Bovine Plasma in Vitro. A plasma pool was prepared from blood taken from steers as described previously.[13] Aliquots were stored frozen at –20 °C and thawed just before use. Three independent experiments were carried out for each analog. In each experiment, a 0.45-mL bovine plasma aliquot was equilibrated to 37 °C for 15 min in a water bath and spiked with 0.05 mL of 0.3 mM aqueous solution of a given GRF analog (GRF concentration not corrected for peptide content). The mixtures were incubated at 37 °C for up to 48 h, depending on the experiment. GRF/plasma aliquots of 0.1 mL were taken at 0 time and various time intervals, quenched immediately with 2 mL of ice-cold 0.2% TFA, and purified by solid-phase extraction on Sep-pak C$_{18}$ (Waters) as described previously.[13] The lyophilized extracts were redissolved in 0.2 mL of 4 M guanidine hydrochloride in 0.05% TFA for HPLC analysis. The amount of peptide in plasma extracts was determined from the area under HPLC peaks. The percent of a given GRF peptide recovered from plasma after a 1-h incubation at 37 °C (stability index) was calculated relative to the amount of peptide extracted from plasma at time 0 taken as 100%. The following are sampling time intervals for full curve analysis: bGRF(1–44)NH$_2$ and [Leu^{27}]bGRF(1–29)NH$_2$, 0, 5, 10, 20, 30, 45, 60, 120, 240, 360, and 480 min (plasma pool D, for plasma pool A and B the 360 and 480 min time points were omitted); [Ala^{15},-Leu^{27}]bGRF(1–29)NH$_2$, 0, 10, 20, 30, 60, 120, 180, 240, and 360 min (plasma pool B and D, for plasma pool A the 360 and 480 min time points were omitted); [Thr^2,Ala^{15},Leu^{27}]bGRF(1–29)-NH$_2$, [Ile^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ and [Val^2,Ala^{15},Leu^{27}]-bGRF(1–29)NH$_2$, 0, 30, 60, 120, 240, 420, 600, 780, 960, and 1500 min (plasma pool D); [Gly^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, [Ser^2,-Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, and [Thr^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, 0, 60, 120, 240, 360, 480, 600, and 840 min (plasma pool B); [Gly^2,Leu^{27}]bGRF(1–29)NH$_2$, [Ser^2,Leu^{27}]bGRF(1–29)NH$_2$, and [Thr^2,Leu^{27}]bGRF(1–29)NH$_2$, 0, 30, 60, 120, 240, 360, 480, and 600 min (plasma pool B). The means from three experiments for each analog were analyzed by one-way analysis of variance [Statistical Analysis System (SAS, Proc GLM)[36]] with a significant probability level of $p < 0.05$. The full disappearance curves of GRF analogs were constructed from the area under the HPLC peaks at each time point. The model for the nonlinear analysis and half-life determinations were based on first-order reaction kinetics as described.[13]

Metabolite Isolation and Characterization. For metabolite characterization, procedures were scaled-up as follows. For [Ser^2,-Leu^{27}]bGRF(1–29)NH$_2$ or [Ser^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, a 1.0-mg sample of peptide was dissolved in 0.02 mL of water and to this was added 1 mL of bovine plasma at 37 °C. The samples were incubated at 37 °C for 18 h. The incubated sample was quenched with 15 mL of 0.2% TFA and divided into two portions and each of portion was processed on a tandem of preconditioned Sep-pack C$_{18}$ cartridges.[13] Each loaded Sep-pak was then rinsed 4 times with 10 mL of 0.05% TFA (eluates discarded), and the

peptide(s) were extracted with 4 × 1 mL of acetonitrile/0.05% TFA (1:1, v/v). The combined extracts were frozen and lyophilized after the acetonitrile removal in vacuo (Speed-vac vacuum evaporator, Savant). For the isolation of the (3–29) fragment from [Ser^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, the plasma extracts from the analytical samples incubated with plasma from 5 min to 4 h were combined and used for the HPLC separation. Metabolites were isolated by semipreparative HPLC on a 1 × 25 cm 218TP510 Vydac C$_{18}$ (5 μM) column using a 0.1% TFA/acetonitrile solvent system with monitoring at 214 nm. Collected fractions were concentrated in the Speed-vac, frozen, and lyophilized. The same procedures were followed for the isolation of the (3–29) fragment from [Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ except that the incubation time with plasma was 2 h. The isolated materials were characterized by mass spectrometry (LSIMS), amino acid analysis, and Edman degradation.

Growth hormone-releasing activity in vitro was tested either in a bovine anterior pituitary cell culture assay[21] or in a rat anterior pituitary primary cell culture system.[23,24]

Growth Hormone-Releasing Activity in Vivo in Steers. The meal-fed steer model of Moseley et al. was used.[22] Holstein steers (255–290 kg) were housed at 18–20 °C in individual stalls and exposed to 16 h of light and 8 h of darkness (lights on at 6:30 a.m.). Steers were meal-fed once daily at 10 am and trained to consume their total daily feed intake in 2 h. The diet consisted of concentrate and alfalfa hay pellets fed to achieve 0.75 kg/day body weight (bw) gains. Animals were administered a single iv dose of bGRF(1–44)NH$_2$ (standard) or GRF analog as follows. Study 1: a 9 × 9 Latin square design; nine steers; dose, 0.01 nmol/kg bw. Study 2: a 15 × 15 Latin square design; 15 steers; dose, 0.01 nmol/kg bw. [His^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$ was initially included in study 2 but when the study was in progress, the peptide was found not to be homogeneous and was removed from further evaluation. Study 3: a 17 × 17 Latin square design; 17 steers; iv injections of sterile water, 0.02 nmol/kg of [Thr^2,-Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, [Val^2,Ala^{15},Leu^{27}]bGRF(1–29)NH$_2$, [Ile^2,Ala^{15}Leu^{27}]bGRF(1–29)NH$_2$, [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-hGRF(1–29)NH$_2$, or 0.1 nmol/kg of other position 2/Ala^{15}-substituted GRF analogs. In study 3, originally, the His^2,Ala^{15} analog was included but during the time of this in vivo experiment the peptide was found not to be homogeneous and was deleted from further evaluation. In the dose–response experiment, a 10 × 10 Latin square design with 10 steers received sterile water, bGRF(1–44)NH$_2$ (0.025, 0.1, 0.4, or 1.6 nmol/kg), or [Thr^2,Ala^{15},-Leu^{27}]bGRF(1–29)NH$_2$ (0.01, 0.05, 0.1, 0.15, or 0.2 nmol/kg bw). All the treatments were at 8 a.m., 2 h before feeding.[22] Blood (6 mL) was collected at 20-min intervals from 40 min before until 120 min after injection with additional samples collected at 5, 10, and 15 min after injection. In the dose–response experiment, 8-mL plasma samples were drawn, the sampling time was extended to 4 h, and a potency estimate was determined by a parallel-line analysis.[37] Serum obtained from the samples was assayed for GH concentration using a radioimmunoassay.[38] Analysis of variance was applied with treatment, animal, and period as main effects.[38] Treatment means were compared using LSMeans from GLM analysis.[39]

Supplementary Material Available: Results of PDMS and amino acids analyses on the synthetic peptides used in the study, Tables VIII–X (4 pages). Ordering information is given on any current masthead page.

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