Evaluation of the Biological Potency of New Agmatine Analogs of Growth Hormone-Releasing Hormone in the Bovine (43401)

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Abstract. **Four new growth hormone-releasing hormone (GHRH) analogs with C-terminal agmatine were compared with the parent human GHRH(1-29)NH_2 fragment to assess their abilities to increase serum concentrations of growth hormone (GH) in the bovine. The four analogs were: [D-Ala^2, Nle^{27}] GHRH(1-28)Agm (JG-73); [desNH_2-Tyr^1, Ala^{15}, Nle^{27}] GHRH(1-28)Agm (MZ-2-51); [desNH_2-Tyr^1, Ala^{15}, D-Lys^{21}, Nle^{27}] GHRH(1-28)Agm (MZ-2-75); and [desNH_2-Tyr^1, D-Lys^{12,21}, Ala^{15}, Nle^{27}] GHRH(1-28)Agm (MZ-2-87). The special characteristic of all four GHRH analogs is that arginine was replaced by agmatine (Agm) in Position 29. Five pregnant Holstein cows received these peptides subcutaneously at the following doses: 0.0156, 0.0625, 0.25, 1, and 4 μg/kg body wt. Each cow received each analog-dose combination according to a 5 × 5 Greco-Latin square design repeated for the 5-week treatment. Each cow also received saline vehicle only at the end of the 5-week treatment. Blood samples were collected from 30 min before until 360 min after treatment injection. Total area under the GH response curves for the 6-hr sampling period for each dose of each GHRH analog was compared. There was a linear dose-dependent GH release in response to hGHRH(1-29)NH_2 and its four GHRH(1-28)Agm analogs. At the dose of 0.25 μg/kg, two GHRH analogs, JG-73 and MZ-2-75, stimulated greater GH release than hGHRH(1-29)NH_2 ($P < 0.05$). No differences were seen at the two lowest doses, 0.0625 and 0.156 μg/kg. When both total area under the GH response curves and GH peak amplitudes for each treatment were averaged for all doses, JG-73 and MZ-2-75 stimulated greater GH release than hGHRH(1-29)NH_2 ($P < 0.05$). In summary, three GHRH(1-28)Agm analogs, JG-73, MZ-2-75, and MZ-2-51, were found to be 11.8, 11.3, and 6.5 times more potent, respectively, on a weight basis, than hGHRH(1-29)NH_2 in stimulating the release of GH in cows.** [P.S.E.B.M. 1992, Vol 200]

Full biological activity of growth hormone-releasing hormone (GHRH) is present in the first 29 amino acids of the molecule GHRH(1-29)NH_2 (1). Further removal of C-terminal amino acids markedly decreases the growth hormone-releasing activity (1-3). It has also been demonstrated that the complete natural molecule hGHRH(1-44)NH_2 and its (1-29)NH_2 fragment are equipotent in stimulating growth hormone (GH) release and milk production in dairy cattle (3, 4).

To enhance its biological activity and its potential applications in clinical medicine and animal husbandry, modifications and substitutions with the corresponding D-isomer amino acids in the GHRH(1-29) fragment have been shown to produce greater GH-releasing activity than the parent 29-amino acid peptide in rats (5-7). Specifically, it has been shown that substitution of Tyr at Position 1 by desNH_2-Tyr^1 or Ala at Position 2 with D-Ala^2, led to GHRH analogs with increased GH-releasing activities as a result of enhanced resistance of the N terminus of the analogs to enzymatic degradation by the aminopeptidases (5, 8). Newly synthesized analogs of GHRH containing 28 amino acids and a C-terminal agmatine (Agm), a decarboxylated arginine substituted in Position 29 instead of arginine, enhance protection against enzymatic degradation at

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the C terminus of the analogs and are over 30 times more potent than the parent hGHRH(1-29)NH_2 after subcutaneous administration in rats (9–11). Previous studies in our laboratory have shown that the administration of a GHRH Agm analog ([D-Ala^2, Nle^{27}] GHRH(1-28)Agm [JG-73]) stimulated greater GH release than the native hGHRH(1-44)NH_2 in the bovine (12).

The objective of this study was to evaluate the biological potency of three new GHRH(1-28)Agm analogs (MZ-2-51, MZ-2-75 and MZ-2-87) compared with the previously tested analog JG-73 and the parent hGHRH(1-29)NH_2 fragment in their ability to increase serum concentrations of GH in the bovine.

Materials and Methods

Animals and Treatments. Five 90-days pregnant, nonlactating Holstein cows, averaging 670 ± 24 kg body wt, were maintained indoors in individual pens and exposed to a 15:9-hr light:dark photoperiod. Water was allowed *ad libitum* and a pelleted ration was fed once daily at 1600 hr. To evaluate health, the body temperature of each cow was checked every week and remained normal throughout the study. Body weight was recorded weekly to calculate the hormone dose for each cow.

Human GHRH(1-29)NH_2 and its four analogs (JG-73, MZ-2-51, MZ-2-75, and MZ-2-87) were synthesized by solid-phase methods and repurified by high-performance liquid chromatography (11). The purity of hGHRH(1-29)NH_2 and its four analogs was 95%. The amino acid substitutions and modifications in the four hGHRH(1-29) analogs were as follows: [D-Ala^2, Nle^{27}] GHRH(1-28)Agm (JG-73); [desNH_2-Tyr^1, Ala^{15}, Nle^{27}] GHRH(1-28)Agm (MZ-2-51); [desNH_2-Tyr^1, Ala^{15}, D-Lys^{21}, Nle^{27}] GHRH(1-28)Agm (MZ-2-75); and [desNH_2-Tyr^1, D-Lys^{12,21}, Ala^{15}, Nle^{27}] GHRH(1-28)Agm (MZ-2-87). The common characteristic of these four GHRH analogs is that arginine in Position 29 was replaced by a decarboxylated arginine, agmatine, to enhance protection against enzymatic degradation at the C terminus of the analogs and methionine^{27} was replaced with norleucine in order to avoid oxidative inactivation (11). All doses of each of the four GHRH(1-28)Agm analogs (JG-73, MZ-2-51, MZ-2-75, and MZ-2-87) and hGHRH(1-29)NH_2 were dissolved in 0.1 *M* CH_3COOH-saline solution and stored at 4°C until time of injection.

Each day, each cow received one subcutaneous injection of hGHRH(1-29)NH_2 or one of its analogs (in the shoulder region) at one of the following five doses: 0.0156, 0.0625, 0.25, 1, or 4 μg/kg body wt, according to a 5 × 5 Greco-Latin square design repeated for 5 consecutive weeks (13). Each cow received each analog-dose combination. At the end of the 5-week period, an additional day was added when each cow received saline vehicle only.

Blood Sampling and Radioimmunoassay. Indwelling jugular catheters were implanted on the day prior to treatment and were maintained for withdrawal of blood samples. Blood sampling began at 7:30 AM on each treatment day. Blood samples were collected via jugular catheters at −30, −15, 0 (injection time), 15, 30, 60, 120, 180, 240, and 360 min. Cannulas were flushed after each sample collection and were filled between blood sampling periods with physiological saline (0.9% NaCl, 1% antibiotic [Oxy-Tet 100; Anchor Division, St. Joseph, MO], and 10 units/ml of heparin). Blood samples were allowed to clot at 4°C for 24 hr. Serum was separated by centrifugation and stored at −20°C until assayed for GH by a previously validated radioimmunoassay (14). Interassay and intra-assay coefficients of variation were 13% and 6%, respectively.

Statistical Analysis. The area under the GH response curve for the entire 6-hr period was determined for each cow at each treatment-dose combination using the trapezoidal summation method (15). Data were analyzed by analysis of variance using general linear models procedures (16) for a repeated Greco-Latin square design with cow, dose and treatment (analog), and days as factors. Because of the design of this experiment, it was impossible to test for a dose and analog interaction in the analysis. Comparisons among treatment means were made by Duncan's multiple range test (17). Orthogonal contrasts were used to determine whether there was a linear, quadratic, or cubic dose-response effect. Potency of analogs, based on total area under the GH response curve, was calculated by the factorial analyses of Bliss and Marks with 95% confidence limits (18).

Results

GHRH(1-29)NH_2 and its four Agm analogs stimulated GH release in the cows as measured by the area under the GH response curve for all treatments (Fig. 1). There was a linear dose-dependent GH release for all treatments ($P < 0.05$). Quadratic and cubic effects were not significant. At the two lowest doses (0.0625 and 0.0156 μg/kg), the GH-releasing activity for the four Agm analogs and GHRH(1-29)NH_2, based on the area under the GH curve, were not different ($P > 0.05$) from one another. At the 0.25 μg/kg dose, MZ-2-75 and JG-73 analogs stimulated greater GH release than GHRH(1-29)NH_2 or analogs MZ-2-51 and MZ-2-87 ($P < 0.05$). At the highest dose of 4 μg/kg, JG-73, MZ-2-75, and MZ-2-51 stimulated greater GH release than the parent GHRH(1-29)NH_2 or analog MZ-2-87 ($P < 0.05$). When treatments were averaged over all five doses, JG-73 and MZ-2-75 stimulated greater GH release than the parent GHRH(1-29)NH_2 ($P < 0.05$). There was no difference in GH-releasing activities

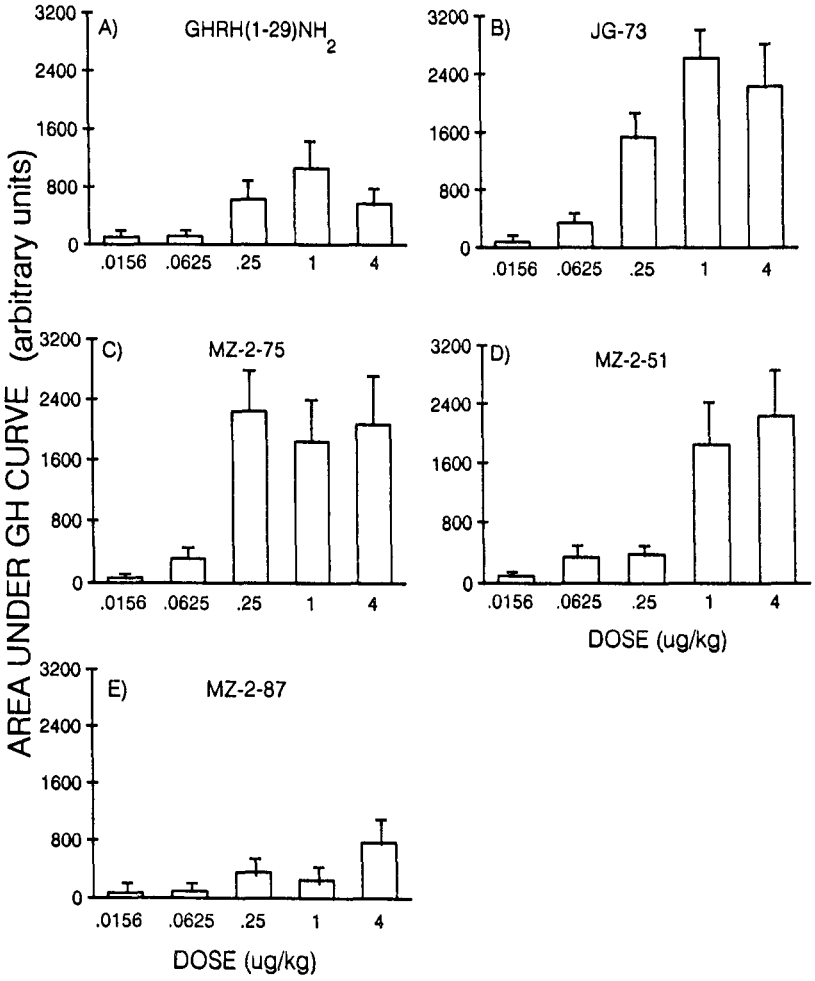

Figure 1. Dose-response effects to hGHRH(1-29)NH_2 and its agmatine analogs (JG-73, MZ-2-75, MZ-2-51, and MZ-2-87) on area under GH response curves for 6 hr after injection. The doses tested were: 0.0156, 0.0625, 0.25, 1, and 4 μg/kg. The results represent the mean and SE for the five cows in each analog-dose combination. The best fit line as determined by linear regression is also included for each analog treatment. The area under GH response curve after saline administration was 11.9 ± 1.8 arbitrary units (mean ± SE for the five cows) and was not represented in this figure because of its small value.

between MZ-2-51 or MZ-2-87 and the parent GHRH(1-29)NH_2 ($P > 0.05$). Furthermore, when GH peak amplitudes for each treatment were averaged for all five doses, JG-73 and MZ-2-75 caused greater GH peak amplitudes than the parent GHRH(1-29)NH_2 or MZ-2-87 analog ($P < 0.05$). Analogs JG-73, MZ-2-75, and MZ-2-51 were calculated to be 11.8, 11.3, and 6.5 times more potent, respectively, than hGHRH(1-29)NH_2 in stimulating the release of GH in cows.

The total area under the GH response curves stimulated by the highest dose (4 μg/kg) of analogs resulted from a biphasic release of GH (Fig. 2). The primary release of GH occurred within 30 min after the analog injection; a secondary release of GH was seen 120–360 min later. The biphasic release of GH was seen only with analogs and not with the parent GHRH(1-29)NH_2. Only a primary GH release was observed at all the lower doses of treatments.

Discussion

The results of this study indicated that two Agm analogs, JG-73 and MZ-2-75, have greater biological potency (approximately 11 times) compared with the parent hGHRH(1-29)NH_2. The GH-releasing activity of analog MZ-2-51 was lower than that of JG-73 and MZ-2-75, but a significant increase of GH was seen after administration of MZ-2-51 at the highest dose of 4 μg/kg. The increased GH-releasing activity of these analogs compared with the parent hGHRH(1-29)NH_2 is probably the result of an increased receptor affinity. It has been observed that a two-point contact with the C terminus binding sites of the receptor through the amine and the guanidine groups of Agm in the analogs may be more favorable than a three-point contact, as in the case of hGHRH(1-29)NH_2 (11). It has been shown that there is higher binding affinity of GHRH analogs compared with human pancreatic growth hor-

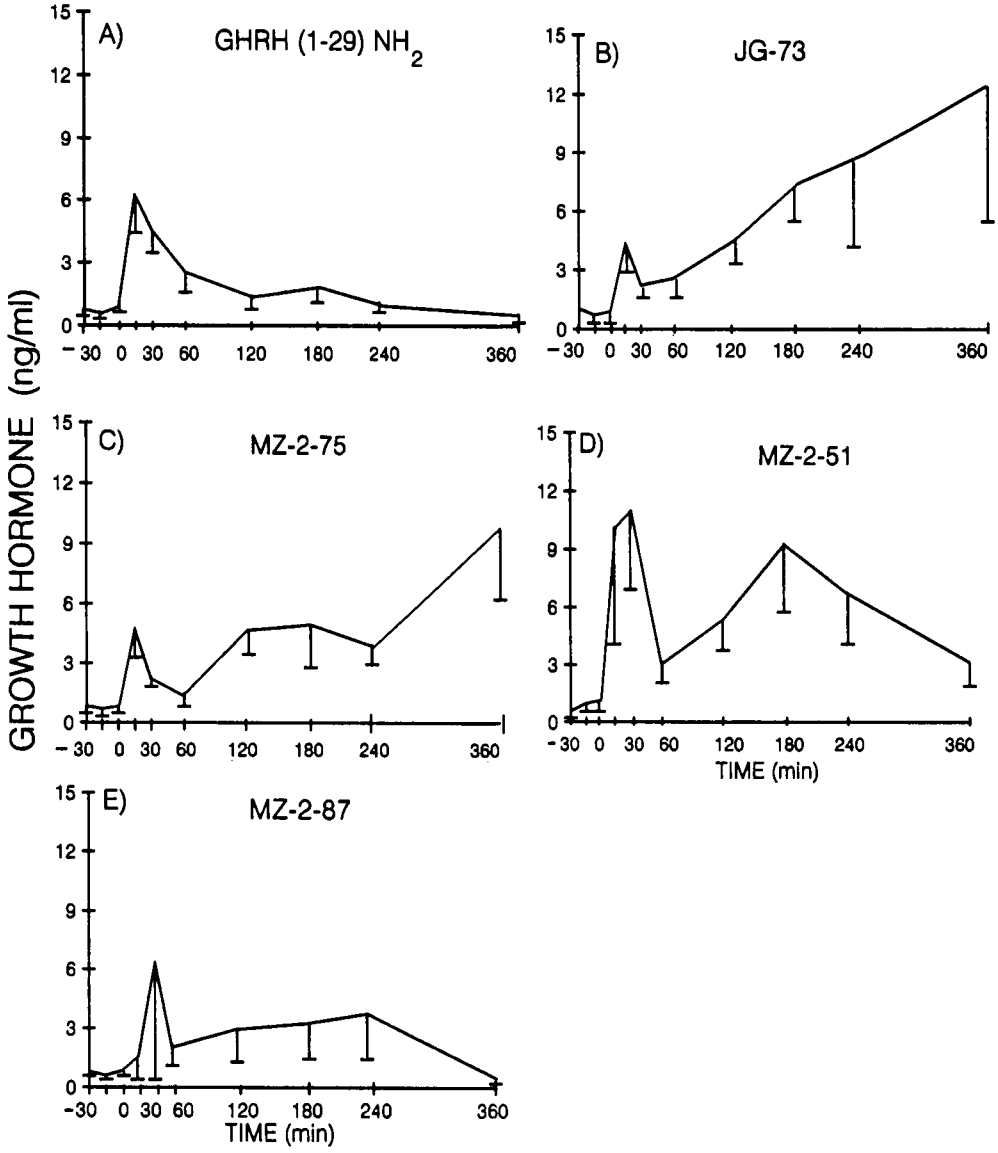

Figure 2. Mean serum GH profiles from cows (n = 5) with the highest dose of 4 μg/kg of GHRH(1-29)NH_2 or its four agmatine analogs (JG-73, MZ-2-75, MZ-2-51, and MZ-2-87) after subcutaneous injection at Time 0.

mone-releasing hormone (hpGHRH[1-40]) in pituitary cell cultures (19).

Another possible reason for the increased GH-releasing activity of these Agm GHRH analogs may be a greater biological stability. The presence of Agm, in place of Arg^{29} in these four GHRH(1-28) analogs, may also increase the resistance to proteolytic degradation at the C-terminal of the analogs (9–11). Moreover, *in vivo* plasma studies have shown that enzymatic degradation of GHRH is initiated by a dipeptidyl peptidase which recognizes the NH_2-terminal Tyr^1-Ala^2 sequence and metabolizes GHRH(1-44)NH_2 (20–22). Since replacement of Tyr^1 by desNH_2-Tyr^1 or Ala^2 by D-Ala^2 resulted in peptides that are poor substrates for dipeptidyl peptidase (21), this could explain why our Agm analogs with the same substitutions at Positions 1 and 2 (as MZ-2-51, MZ-2-75, MZ-2-75, and JG-73) were more biologically stable than the parent hGHRH(1-29)NH_2 fragment. In addition, cleavage can occur at Positions 11–12 or 20–21 of GHRH because these sites of dibasic residues are susceptible to the action of trypsin-like enzyme (21). Therefore, substitutions of D-isomer amino acids at Positions 12 and/or 21 (as seen in analogs MZ-2-75 and MZ-2-87, respectively) have been done to increase resistance to these enzymes. In this study, MZ-2-75 analog, which had one D-Lys^{12} substitution, was 11.3 times more active in its GH-releasing activity than hGHRH(1-29)NH_2. However, analog MZ-2-87, which had D-Lys substitutions at Positions 12 and 21, had decreased GH-releasing activity compared with its parent hGHRH(1-29)NH_2. This same observation was seen in rat studies, in which the

analog MZ-2-87 was the least active in stimulating *in vivo* and *in vitro* release of GH compared with other analogs (11). The same authors concluded that GHRH(1-28)Agm analogs with both D-Lys substituted in Positions 12 and 21 (as in MZ-2-87) were less active than the analogs with only one D-Lys substitution (as in MZ-2-75).

Analogs of GHRH(1-29)NH_2 in which Gly^{15} is replaced by Ala or other hydrophobic amino acids have been shown to have increased potency (8). The increased biological activity for the 15-substituted GHRH analogs may be due to enhanced α-helicity and maximization of the amphiphilic secondary structure of the analogs and may improve their binding to the receptor (8, 23–25). In this study, MZ-2-75 and MZ-2-51 analogs were substituted with Ala^{15} and both had greater GH-releasing activity than the parent hGHRH(1-29)NH_2 fragment, confirming the rat studies. In this study, the GHRH(1-28)Agm analogs with the greatest GH-releasing activities were those that had: (i) one D-Lys sustitution in their molecule (JG-73 and MZ-2-75) and (ii) substitution in Positions 1 and 15 (MZ-2-51). Analogs containing two substituted D-Lys in its molecule displayed decreased GH-releasing activity (MZ-2-87). The biphasic release of GH observed following treatment with GHRH(1-29) analogs has been reported previously in humans (26), steers (27), and heifers (3, 12). In rats, GH is stored within two compartments in the anterior pituitary (28). The primary release of GH occurs from a small pool of GH that is released quickly in response to GHRH, but is rapidly exhausted. The secondary release of GH originates from a larger pool that is released more slowly and continuously in response to long-term stimulation. In the present study, the biphasic response following administration of the highest dose (4 μg/kg) of GHRH(1-28)Agm analogs in cows may represent mobilization and release of stored GH from these compartments of the anterior pituitary.

These GHRH(1-28)Agm analogs have been shown to be over 30 times more active than hGHRH(1-29)NH_2 after subcutaneous injection in rats (9). In our cow study, the most potent Agm analogs (JG-73 and MZ-2-75) were only approximately 11 times more active than hGHRH(1-29)NH_2. This difference in response between cows and rats has also been reported before between humans and rats, and could be caused by a species difference in receptor affinity and (or) rate of enzyme degradation of these peptides (9–11).

A previous study in our laboratory has shown that analog JG-73 administered intravenously was 4.6 times more potent than hGHRH(1-44)NH_2 in stimulating the release of GH in heifers (12). Injected subcutaneously, the same JG-73 analog was 11.8 times more potent than hGHRH(1-29)NH_2. A similar comparison made in rats also found that GHRH(1-28)Agm analogs were more potent when administered subcutaneously versus intravenously (9–11). The ratio of GH-releasing activity between intravenous and subcutaneous administered analogs ranged from 2 to 5 (9). Since our findings in the bovine parallel the rat results, subcutaneous administration of GHRH(1-28)Agm analogs may have better efficacy to cause release of GH.

Conclusion

Three GHRH(1-28)Agm analogs JG-73, MZ-2-75, and MZ-2-51, were found to be 11.8, 11.3, and 6.5 times more potent, respectively, than hGHRH(1-29)NH_2 in increasing GH release in cows. These analogs offer potential application in the induction of GH secretion in domestic livestock for promotion of growth and lactation.

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