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Release of exendin-4 is controlled by mechanical action in Gila Monsters, *Heloderma suspectum*

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Abstract

Exendin-4 is a peptide produced exclusively by the salivary glands of the Gila Monster, *Heloderma suspectum*. Although exendin-4 is considered a venom component, circulating plasma levels of exendin-4 have been shown to increase in response to feeding. Previous studies using mammals have demonstrated exendin-4 has prolonged plasma glucose-lowering properties. While these findings suggest a possible role of exendin-4 as a metabolic hormone in the Gila Monster, the mechanism controlling its release by the salivary gland has not previously been studied. We investigated possible factors driving exendin-4 release by testing Gila Monsters' response to one of six treatment groups: fed egg, fed juvenile rat, gastric intubation with egg while under anesthesia, olfactory stimulation from egg without ingestion, unfed control, and biting without feeding. These treatments were designed to separately test actions associated with feeding and different food types. We measured plasma exendin-4 levels using an immunoenzymetric assay before and at three time points after each treatment. Exendin-4 levels increased significantly in groups where considerable biting occurred but not in the other treatment groups. These results suggest that exendin-4 is released from the salivary glands in response to mechanical stimulation and not the detection of food either by smell, taste, or distention of the gut. Further study of exendin-4 in its natural organism is needed to elucidate the functional role of exendin-4 as a venom component and/or a metabolic regulator.

Keywords: Exendin-4; Food-forward release; Gila Monster; *Heloderma suspectum*; Metabolic hormone; Novel protein; Reptile; Venom

1. Introduction

Exendin-4 is a 39-amino acid peptide unique to the saliva of the Gila Monster, *Heloderma suspectum* (Eng et al., 1992; Chen and Drucker, 1997). Exendin-4 has a high affinity for GLP-1 receptors and has prolonged blood glucose-lowering properties in both mice and humans. These results have generated considerable research exploring the possible use of exendin-4 as a diabetes treatment. Despite its popularity as a potential therapeutic agent, exendin-4 remains poorly studied in its native organism. While exendin-4 was initially considered a venom component used in the acquisition of prey, Young et al. (1999a), in the only study to date on exendin-4 in Gila Monsters, demonstrated a rapid and dramatic postprandial increase in plasma exendin-4 levels, which suggests a physiological role of exendin-4 in Gila Monsters. Thus, we wanted to further explore the potential physiological role of exendin-4 in the Gila Monster. Specifically, we examined how various stimuli associated with prey acquisition and digestion affect the release and subsequent elevated plasma levels of exendin-4 in Gila Monsters.

The Gila Monster provides an exceptional model for exendin-4 studies not only because it is the natural source of this peptide, but also because Gila Monsters naturally eat very large meals at infrequent intervals. Gila Monsters primarily consume reptile and bird eggs, hatchlings, or newborn rabbits and rodents and can ingest prey weighing 1/3 of their body mass in one meal. At the other extreme, Gila Monsters can go months without feeding (Beck, 2005). Such a diet must lead to dramatic shifts in digestive and metabolic physiology and likely requires an extraordinary ability to regulate digestive processes. This physiological challenge may have led to the evolution of exendin-4 in Gila Monsters. Due to its rapid increase in plasma after feeding and GLP-1 receptor binding

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properties, exendin-4 may regulate various digestion and absorption-related events.

A postprandial elevation in circulating exendin-4 levels could be a result of secretory stimuli associated with prey detection, prey apprehension, and/or digestion/absorption. Due to its origin in the salivary glands, we hypothesized that mechanical events associated with prey apprehension provided the greatest stimulus for exendin-4 release. To test our hypotheses, we created experimental groups that isolated each of the major components of predation—detection, apprehension, and digestion.

2. Materials and methods

We divided 36 captive Gila Monsters into six treatment groups ($n=6$) designed to test various aspects associated with feeding: (1) unfed (negative control, CON), (2) fed 60 ml egg gelatin (positive control liquid meal, EGG), (3) smelled egg with resultant tongue-flicking, but not allowed to feed (prey detection stimulus only, SMELL), (4) fed rat pups (positive control solid meal, RAT), (5) fed 60 ml egg gelatin by intragastric intubation under anesthesia (digestion stimulus only, INTUBATED), and (6) chewed a padded stick but not fed (apprehension stimulus only, CHEW). Treatments were administered randomly over an 8-day period and at an ambient temperature of 26 °C. For the EGG group, we placed Gila Monsters individually in a plastic box (36 × 52 × 16 cm) with a shallow bowl containing 60 ml of prepared egg gelatin (two eggs mixed with one package plain gelatin and chilled to set) and allowed them to feed freely until the egg gelatin was consumed (less than 8 min). Gila Monsters in the SMELL group were treated similar to those in the EGG group except that the shallow bowl contained 10 ml of raw egg and was covered by a fine mesh which prevented the Gila Monsters from eating the egg. Gila Monsters in the SMELL group frequently tongue-flicked the top of the bowl while trying to get to the egg. For the RAT group, we placed Gila Monsters individually in a similar plastic box with three 12-day (approximately 20 g) rat pups, which they consumed within 5 min. For the INTUBATED group, we placed each Gila Monster into an anesthesia chamber containing isoflurane until it was not responsive to touch. At that point, an endotracheal tube was inserted and anesthesia was maintained with 2% isoflurane. Once a surgical plane of anesthesia was established, we fed the Gila Monster 60 ml of egg gelatin through an intragastric tube. While limitations on animal availability prevented us from including a negative control for the effect of anesthesia on exendin-4 level, a preliminary study demonstrated that anesthesia does not alter circulating exendin-4 levels (unpublished data). For the CHEW group, we presented Gila Monsters with a padded flat stick and coerced them to bite repeatedly on the stick for 3 to 5 min. For all treatment groups, we took blood samples from each animal before any treatment (time 0) and 15 min, 45 min, and 24 h after treatment. Blood samples were collected from the caudal tail vein using heparinized 1 cm^3 syringes. For the INTUBATED group, the first three samples were taken while the animals were at a surgical plane of anesthesia. Within 15 min of collection, we separated plasma from whole blood via centrifugation and stored it at −80 °C until analysis by Amylin Pharmaceutical for exendin-4 levels.

Plasma exendin-4 was measured using a two-site sandwich immunoenzymetric assay (IEMA) with fluorescent detection. Microtiter plates were coated with monoclonal antibody EXE4: 2–8.4 (EXE4: 2–8 recognizes a c-terminal epitope on exendin-4 and does not cross react with GLP-1 or glucagon) by adding 50 μL (5 μg/mL) to each well and incubated overnight at 2–8 °C. Wells were then washed with TBS/Tween (0.05 M Tris, 0.15 M sodium chloride, 0.02% sodium azide, 0.1% Tween-20) and blocked using 1% nonfat dried milk diluted in carbonate buffer (0.05 M, pH 9.5). Specimens, standards, and plasma controls were thawed on ice and, if required, diluted in Plasma Mimic (4% Bovine Serum Albumin, 200 mg/dL bovine cholesterol supertrate). Specimens, controls and standards (50 μL) were then added to the plates and incubated for 1–2 h at room temperature. After washing the plates with TBS/Tween (0.05 M Tris, 0.15 M sodium chloride, 0.02% sodium azide, 0.1% Tween-20), 50 μL of biotinylated monoclonal detection antibody GLP1:3–3.1 (GLP1:3–3 recognizes a n-terminal epitope on both exendin-4 and GLP-1) pre-incubated with streptavidin-alkaline phosphatase was added. After a 1–2-h incubation at room temperature, plates were washed thoroughly with TBS/Tween-20 followed by a TBS wash without Tween-20. A signal was generated by the addition of 50 μL of a solution containing alkaline phosphatase substrate 4-methylumbelliferyl phosphate (4-mup). After incubation, the reaction was stopped by the addition of 50 μL of a solution containing 0.1 M sodium phosphate and 1.5 M sodium chloride. The fluorescent signal was detected with a microplate fluorometer and data analyzed with StatLia software using the 4-parameter fit option. Concentrations of exendin-4 in plasma samples were determined by comparison with a calibration curve run with the same assay. Using SAS, we then analyzed differences in exendin-4 level among groups and over time using repeated measures analysis of variance (RMANOVA, $\alpha=0.05$) with treatment group as the between-subjects factor, time as the within-subjects factor, and exendin-4 level as the dependent variable.

3. Results

The RMANOVA of exendin-4 levels revealed significant effects between treatment groups ($F_{5,30}=13.36$, $p=<.001$) and over time ($F_{3,30}=14.48$, $p=<.001$), as well as a time by treatment group interaction ($F_{15,30}=7.66$, $p=<.001$). Post-hoc analyses revealed the CHEW and RAT groups had significantly elevated exendin-4 levels at 15 and 45 min post-treatment (Fig. 1, Table 1). At these time points, the CHEW group had the greatest increase in exendin-4 level and was significantly higher (an approximate four-fold greater increase) than the RAT group exendin-4 level. However, exendin-4 levels between the CHEW and RAT groups were not significantly different from the other treatment groups before treatment or 24 h after treatment. Post-treatment plasma exendin-4 levels in the CON,

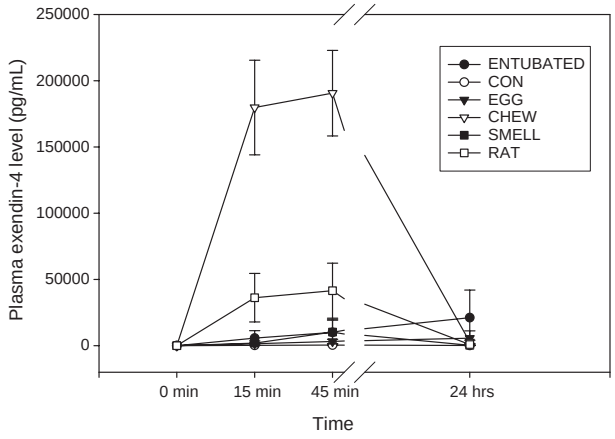

Fig. 1. Increase in plasma exendin-4 levels over time in response to various feeding treatments. Mean values and standard error reflect the differences from initial values (time 0). The CHEW and RAT groups had significantly higher plasma exendin-4 levels 15 and 45 min after treatment. For all groups, plasma exendin-4 level at 24 h did not differ from baseline.

EGG, INTUBATED, and SMELL treatment groups were not significantly different from each other or from initial values (Fig. 1, Table 1).

4. Discussion

This study clearly demonstrates that exendin-4 is released from the salivary glands in response to mechanical action (movement of the mouth) as the CHEW and RAT groups were the only groups where significant chewing occurred and the only groups with elevated plasma exendin-4 levels. The lack of a response in the other treatment groups suggest that smell, taste, and stomach distention are not effective stimuli for exendin-4 release. Although subjects in the EGG group moved their mouths to ingest the egg gelatin, the movement was limited compared to the CHEW or RAT groups as the egg was ingested primarily by licking. The significant difference in plasma exendin-4 levels between the CHEW and RAT groups is most likely due to the greater incidence of chewing in the CHEW group. Subjects in the CHEW group were coerced to continually bite on the padded stick for at least 3 min. However, the RAT subjects were not continually biting on the rat pups while restraining and ingesting them. Therefore, even though the treatment time was similar between the CHEW and RAT groups, the amount (and likely the intensity) of mastication was greater in the CHEW group, thus resulting in higher exendin-4 levels.

Since considerable mechanical action of the mouth occurs during a defensive bite and during apprehension of relatively large prey, our results do not distinguish whether the primary role of exendin-4 serves in defense, prey apprehension, or energy processing. If exendin-4's main function is as a venom protein used for prey apprehension, it should have deleterious consequences when injected into the prey. However, when injected into laboratory mice, a species similar to natural Gila Monster prey, exendin-4 has no effect in normal mice and only glucose-lowering properties in diabetic mice (Young et al., 1999b). Such effects would not aid a Gila Monster in apprehending or killing its prey. Human clinical trials with exendin-4 have demonstrated physiological effects similar to those in mice (Egan et al., 2002). Since humans represent a predatory threat to Gila Monsters, the lack of any deterring effect of exendin-4 suggests that this protein has limited value for defense.

The lack of any detectable effect of exendin-4 as a venom component (for prey acquisition or defense) suggests that this protein may have a role in regulating Gila Monster physiology (i.e., it functions as a hormone). This possibility is supported by the presence of exendin-4 in the bloodstream following the consumption of a large meal (Young et al., 1999a). However, salivary glands, where exendin-4 is produced, are not known to have an endocrine function in vertebrates, and proteins in general have limited ability to cross cell membranes (a route that would be necessary if exendin-4 is simply secreted into the buccal cavity and absorbed into the bloodstream). Therefore, the rapid and dramatic increase in plasma exendin-4 levels post-prandially is intriguing. Interestingly, systemic absorption of the closely related peptide GLP-1 occurs when it is delivered orally as a microsphere or a mucoadhesive, biodegradable tablet (Gutniak et al., 1997; Joseph et al., 2000). However, the bioavailability of GLP-1 via this route is only 6% relative to intravenous administration (which would be similar to an endogenous endocrine route) (Gutniak et al., 1997). While this may explain exendin-4's rapid increase in plasma, future studies on the mechanisms of release of exendin-4 into the blood are warranted.

Of even greater value would be studies on the metabolic effects of circulating exendin-4 in Gila Monsters. Our results indicate that exendin-4 is released after ingestion of large meals, and thus, if exendin-4 does have a role in metabolism, the role may be focused on the digestion of foods requiring lengthy pre- or post-absorption processing (i.e., food-forward release). Gila Monsters are binge feeders, consuming very large meals at infrequent intervals. Recent studies of other binge-

Table 1
Mean plasma exendin-4 values (pg/mL±1 S.E.M.) with minimum and maximum values in parentheses for each treatment group ($n=6$)

	0 min	15 min	45 min	24 h
CON	134±48 (50, 362)	350±107 (63, 769)	608±171 (142, 1164)	227±159 (50, 860)
EGG	5044±4628 (79, 28170)	6311±4357 (173, 27610)	8199±6191 (204, 38920)	552±223 (130, 1523)
SMELL	214±88 (50, 538)	2181±1727 (50, 10750)	10520±8904 (165, 54850)	267±102 (72, 591)
INTUBATED	18307±17780 (171, 107300)	23936±23433 (153, 141100)	8374±7820 (218, 47460)	1003±463 (360, 2810)
RAT	297±127 (100, 870)	36522±18342 (2630, 101000)	41810±20742 (3860, 130000)	1157±428 (160, 2620)
CHEW	2613±2127 (57, 13170)	182390±36586 (33390, 250000)	193220±33064 (47270, 250000)	4110±1848 (399, 8705)

feeding reptiles, particularly pythons, have demonstrated that considerable physiological changes are needed to process large meals. Large meal consumption leads to dramatic changes in gastrointestinal anatomy and physiology (Lignot et al., 2005; Secor, 2003; Secor et al., 2001) as well as cardiovascular anatomy and physiology (Andersen et al., 2005). However, little is known about changes needed to process large concentrations of absorbed nutrients in these binge-feeders. Studies of Gila Monsters may provide an alternative model for examining these effects and thus contribute to the general understanding of how the body deals with shifts in energy availability.

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