Circulating Glucagon-like Peptide-1 (GLP-1) Inhibits Eating in Male Rats by Acting in the Hindbrain and Without Inducing Avoidance

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To address the neural mediation of the eating-inhibitory effect of circulating glucagon-like peptide-1 (GLP-1), we investigated the effects of 1) intra-fourth ventricular infusion of the GLP-1 receptor antagonist exendin-9 or 2) area postrema lesion on the eating-inhibitory effect of intrameal hepatic portal vein (HPV) GLP-1 infusion in adult male rats. To evaluate the physiological relevance of the observed effect we examined 3) the influence of GLP-1 on flavor acceptance in a 2-bottle conditioned flavor avoidance test, and 4) measured active GLP-1 in the HPV and vena cava (VC) in relation to a meal and in the VC after HPV GLP-1 infusion. Intrameal HPV GLP-1 infusion (1 nmol/kg body weight-5 min) specifically reduced ongoing meal size by almost 40% ($P < .05$). Intra-fourth ventricular exendin-9 (10 μg/rat) itself did not affect eating, but attenuated ($P < .05$) the satiating effect of HPV GLP-1. Area postrema lesion also blocked ($P < .05$) the eating-inhibitory effect of HPV GLP-1. Pairing consumption of flavored saccharin solutions with HPV GLP-1 infusion did not alter flavor acceptance, indicating that HPV GLP-1 can inhibit eating without inducing malaise. A regular chow meal transiently increased ($P < .05$) HPV, but not VC, plasma active GLP-1 levels, whereas HPV GLP-1 infusion caused a transient supraphysiological increase ($P < .01$) in VC GLP-1 concentration 3 minutes after infusion onset. The results implicate hindbrain GLP-1 receptors and the area postrema in the eating-inhibitory effect of circulating GLP-1, but question the physiological relevance of the eating-inhibitory effect of iv infused GLP-1 under our conditions. (***Endocrinology*** **155: 1690–1699, 2014)**

Carbohydrates and fats potently stimulate glucagon-like peptide-1 (GLP-1) (GLP-1-(7–37) and GLP-1-(7–36)-amide) release from enteroendocrine L-cells. GLP-1 enters either intestinal lymph ducts or capillaries draining into the hepatic portal vein (HPV) (1–4). Dipeptidyl peptidase-4 rapidly degrades circulating GLP-1 to inactive forms, limiting its biological half-life in the blood to about 3 minutes (5, 6). GLP-1 and its potent, long acting analogs (eg, exendin-4 [Ex-4], liraglutide) inhibit eating in animals (7–13) and humans (14) and have produced promising weight-loss and antidiabetic effects in clinical trials. Nevertheless, a better understanding of the mechanisms underlying GLP-1's effects is essential before widespread use of GLP-1-based treatments can be recommended (14).

Brief, intrameal peripheral HPV, inferior vena cava (VC), and ip GLP-1 infusions (1 nmol/kg body weight [BW]) selectively reduced spontaneous meal size in rats (15). The effect of ip, but not HPV GLP-1, required intact abdominal vagal afferents, suggesting that circulating

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Abbreviations: AP, area postrema; APS, area postrema sham; APX, area postrema lesion; Arc, hypothalamic arcuate nucleus; AUC, area under the curve; BW, body weight; CeA, central area of the amygdale; CFI, cumulative food intake; CS−, conditioned stimulus-negative flavor; CS+, conditioned stimulus-positive flavor; Ex-4, exendin 4; Ex-9, exendin 9; Exp, experiment; GLP-1, glucagon-like-peptide-1; GLP-1R, glucagon-like-peptide-1 receptor; HPV, hepatic portal vein; IC4V, intra-fourth ventricular; ID, inner diameter; IMI, intermeal interval; NTS, nucleus tractus solitarii; OD, outer diameter; PVN, hypothalamic paraventricular nucleus; SED, standard error of difference, 5TG, 5-thio-glucose; VC vena cava.

GLP-1 acts in the brain to inhibit eating (15). The central nervous system mechanisms mediating this action, however, are uncertain. GLP-1 receptors (GLP-1Rs) are expressed in several brain areas implicated in eating control, including the hypothalamic paraventricular (PVN) and arcuate nuclei (Arc), the central area of the amygdala (CeA), the nucleus tractus solitarii (NTS), and the area postrema (AP) (16, 17). Studies using radiolabeled GLP-1 revealed that AP GLP-1Rs are accessible to circulating GLP-1 (18). Using c-Fos immunocytochemistry to map neuronal activation, we found that HPV GLP-1 infusion induced c-Fos expression in the NTS, AP, and CeA, but not the PVN or Arc (19), suggesting that circulating GLP-1 inhibits eating by activating hindbrain GLP-1Rs. We here tested this hypothesis by determining whether infusion of a GLP-1R antagonist into the fourth ventricle (Experiment [Exp] 1) or AP lesion (APX; Exp 2) reduces the eating-inhibitory effect of remotely controlled, intrameal HPV GLP-1 infusion. Exogenous GLP-1 can elicit malaise. When exactly GLP-1 produces satiation or malaise, and the relative contributions of peripheral and central GLP-1R to these effects, are unclear. Central GLP-1 infusions produced robust avoidance (20), and central nervous system GLP-1R activation (21, 22), including CeA GLP-1R (23–25), may contribute to the aversive effect of LiCl (21, 22). GLP-1R in the CeA may mediate some of the avoidance response to GLP-1 (23–25). Because HPV GLP-1 infusion increased c-Fos expression in the CeA (19), we tested (Exp 3) whether GLP-1 induces flavor avoidance under our conditions. Finally, to examine the physiological relevance of the eating inhibition after HPV GLP-1 infusion under our conditions, we measured changes in plasma levels of GLP-1 in response to regular chow meals (Exp 4) and HPV GLP-1 infusion (Exp 5).

Materials and Methods

Animals and housing

Male Sprague Dawley rats (Crl:CD; Charles River) were kept individually in a temperature-controlled (21 ± 2°C) room with a 12-hour light/12-hour dark cycle and ad libitum access to tap water and chow (catalog no. 3436; Provimi Kliba NAFAG) except where otherwise noted. After postsurgical recovery, the rats were housed individually in custom-made acrylic infusion cages with stainless-steel grid floors and adapted to the experimental procedures. Ground chow (Kliba; catalog no. 3433) was presented in food cups mounted on electronic balances (PM3000; Mettler Toledo) under a niche about 6 cm above the cage floor. The balances were interfaced with a computer in the adjacent room, and a custom-designed program (VZM Krügel) recorded food-cup weights every 30 seconds for meal pattern analysis. Infrared video cameras (VSS3440; Philips) permitted continuous remote surveillance of the rats. Standard pre- and postsurgical anesthetic medication and antibiotic prophylaxis were given for all surgeries, as described previously (15, 26). Rats had ad libitum access to food and water for the recovery period, and BW was monitored daily. All procedures were approved by the Canton of Zurich Veterinary Office.

Surgeries

General

Inhalation anesthesia for the fourth ventricular cannulation (IC4V, Exp 1) and HPV and VC catheterizations (Exp 4 and Exp 5) was initiated with 5% isoflurane (Attane, Minrad Inc) in oxygen and maintained with 2%–3% isoflurane in oxygen (Conoxia, Pan Gas). Anesthesia for other surgeries was induced by ip injection (1.1 mL/kg) of a freshly prepared mixture of 80 mg/kg ketamine (Narketan, Vetoquinol AG) and 4.0 mg/kg xylazine (Rompun 2%, Bayer). Additional ip doses of 0.1 mL ketamine were administered if necessary.

IC4V cannulation

The skull was fixed in a stereotaxic apparatus (David Kopf Instruments) and a 26 G stainless-steel guide cannula (model C315G; PlasticsOne) was implanted above the fourth ventricle (2.5 mm anterior to occipital suture line, and 5.0 mm below the surface of the skull) (27, 28). The guide cannula was fixed with dental acrylic cement to 3 stainless-steel screws anchored to the skull and closed with a removable obturator (model C315DC, PlasticsOne) that protruded 1 mm below the guide-cannula tip.

Cannula placement was verified 5 days before the experiment by infusing 5-thio-glucose (5TG), a nonmetabolizable glucose analog that acts in the NTS to trigger a hyperglycemic response (29). Rats fasted for 1 hour were infused (90 seconds) with 210 μg 5TG (MT04882; Carbosynth) dissolved in 3 μL sterile saline (NaCl 0.9%; Braun Medical AG). The 33 G stainless-steel injector (model C315IC) extended 2 mm beyond the guide cannula and was connected via SILASTIC tubing (Becton Dickinson and Company; inner diameter [ID], 0.51; outer diameter [OD], 0.93 mm) to a 10 μL microsyringe mounted on a micropump (Ultramicropump III; World Precision Instruments). Following the infusion, the injector cannula was kept in place for 30 seconds to avoid infusate backflow. Glucose was measured in HPV blood with a glucometer (Accu-Chek; Roche Diagnostics). The criterion for correct IC4V cannula placement was a 100% increase over baseline blood glucose at 60 minutes after the IC4V 5TG. In rats that failed to meet this criterion, the test was repeated after 2 days with the injector extending 2.5 mm beyond the guide cannula. All subsequent infusions were performed using the same injector length as in the functional test. Another 5TG test was done after the experiment. Only data from animals that passed both tests were included.

APX

APX was performed as previously reported (30). Briefly, the head was fixed in a stereotaxic head holder and ventroflexed, the skin was opened from rostral to the occipital crest to the mid-cervical level, and the cervicoauricular and splenius muscles were bluntly dissected and carefully retracted laterally. Under a surgical microscope the atlanto-occipital membrane and meninges were incised at their insertion on the base of the occipital bone to expose the dorsal medulla. The AP was visualized and gently

aspirated by suction with a blunt 23 G needle attached to a vacuum line. AP sham (APS) surgery was performed identically, except the AP was not aspirated.

Five weeks after surgery, APX were functionally verified by measuring spontaneous hypertonic saline intake, which is substantial after APX, but mimimal in intact animals (31, 32). Rats had ad libitum access to 0.5 M NaCl and tap water in preweighed bottles. The inclusion criterion for successful APX was consumption of more than 25 mL saline in 24 hours. After the experiment, the rats were euthanized, the brains were prepared histologically, and sections from the entire lesion area were examined microscopically by 2 investigators blind to the behavioral data to identify animals in which the AP was removed and the surrounding NTS tissue appeared largely intact (Supplemental Figure 1). Only results from rats that passed the functional test and had histologically confirmed APX were included.

HPV and VC catheterization

In Exp 1 and Exp 2, HPV catheters were implanted 9 days (Exp 1) or 3 weeks (Exp 2) after IC4V and APX surgeries. Catheters made from SILASTIC tubing attached to U-shaped stainless steel ports of 22 G for HPV (20 G for infusion experiments) and VC were assembled as described previously (33, 34) and implanted using a sterile technique. HPV and VC catheters were flushed with 0.2 mL of 0.9% sterile saline (NaCl 0.9%; Braun Medical AG) and filled with 80 μL of heparinized saline (100 IU heparin/mL saline; heparin; Braun) daily for the first week after surgery and every alternate day thereafter (for detailed descriptions see References 15, 33, and 35).

Successful blood withdrawal was considered proof of patency and correct placement of HPV/VC catheters. If blood could not be aspirated in Exp 1–Exp 3, 0.8 mL/kg BW of a mixture of 35.6 mg/kg ketamine (Narketan) and 0.9 mg/kg xylazine (2% Rompun) was infused into the HPV after the behavioral tests. The criterion for catheter patency was a complete loss of muscle tone within 1 minute. In addition, 0.5 mL of Giemsa stain (Sigma-Aldrich) was infused into the catheters after postmortem laparotomy, and the perfusion of the HPV or VC was visually verified. Only data from rats with catheter patency confirmed by both the blood-withdrawal or the anesthesia test and the postmortem tests were included.

HPV GLP-1 infusions

In Exp 1–3 and 5, 1 nmol/kg BW GLP-1(7–36)-amide (Bachem FPC0090,) dissolved in PBS (Life Technologies, Inc) with 1% BSA (Sigma-Aldrich) or vehicle (PBS with 1% BSA) was infused (0.2 mL/min $\times$ 5 minutes) into the HPV.

Exp 1: Effect of IC4V exendin-9 (Ex-9) on the satiating effect of HPV GLP-1

Rats (BW 395 $\pm$ 11 g) were adapted to the experimental procedures, including HPV and IC4V vehicle infusions, for 10 days and then tested in a within-subjects crossover design. On test days, food cups were closed 3.5 hours before dark onset. Two to 1.0 hours before dark onset, Ex-9 (10 μg/rat; H-8740; Bachem) dissolved in 2 μL 0.9% sterile saline or vehicle was infused (60 seconds) IC4V, and the HPV catheters were attached to the infusion pumps (A99; Razel) via 2 segments of polyethylene tubing (ID, 0.74 mm; OD, 1.22 mm; Becton Dickinson & Co). The lower segment was sheathed with a stainless-steel spring fixed to a swivel joint 45 cm above the cage floor, allowing the rats to move freely. The IC4V infusion procedure was the same as described for the 5TG test. At dark onset, fresh ground chow was offered. Two minutes after meal onset, HPV infusion of GLP-1 or vehicle was triggered from the control room. Food cup weights and the rats' behavior were constantly monitored. Criteria for meal onset were a decrease of more than or equal to 0.3 g weight and visual verification of eating. Four hours after dark onset, catheters were disconnected from the infusion pumps and flushed. HPV GLP-1/vehicle and IC4V Ex-9/vehicle infusions were tested in random order on 4 separate days with 2 days intervening between tests.

Exp 2: Effect of APX on the satiating effect of HPV GLP-1

APX and APS rats (BW 396 $\pm$ 16 and 450 $\pm$ 15 g, respectively) were tested 4 and 7 weeks after HPV and APX surgery, respectively. The rats were adapted to the procedures for 10 days before the experiment. Intrameal HPV GLP-1 and vehicle infusions were tested in random order on 2 separate days with 2 intervening days.

Exp 3: Effect of HPV GLP-1 on flavor acceptance

Three weeks after HPV catheter implantation, rats (BW 410 $\pm$ 4 g) were adapted to a water-deprivation schedule for 4 days, with water offered 30 minutes in the light phase (2.5–2 hours before dark onset) and 40 minutes in the dark phase (4 hours after dark onset). During the dark-phase access, no food was available from 4–6.5 hours after dark onset to prevent flavor associations with meals. Fluids were offered in 50-mL Falcon tubes (Orange Scientific) fitted with a drinking valve with neoprene stopper (type TD100, Uno Roestvaststaal BV). Separate tubes and nipples were assigned to all fluids to avoid cross-contamination. During adaptation days, rats received HPV vehicle infusions. After adaptation, rats received saccharin solution (0.2%, Sigma Chemical Co) for 2 days in the dark-phase access period to reduce neophobia or to increase the flavor's palatability. The trial comprised 4 days: On association day 1, rats had access to 0.2% saccharin flavored with orange or lemonade (0.05% Kool-Aid; Kraft Foods) during the 40-minute dark-phase access period, followed by HPV infusion of GLP-1; on association day 2, flavors and infusates were reversed. For half of the rats the orange flavor was paired with GLP-1 infusion (= conditioned stimulus positive flavor [CS+]) and the lemonade flavor with vehicle (= conditioned stimulus negative flavor [CS−]). For the remaining rats the CS+ and CS− were reversed. On the intervening day rats received only water. On the evocation day rats received one bottle of each flavor in the dark phase for 40 minutes (Supplemental Figure 2). Drinking-bottle positions were switched in the middle of the session to minimize place preferences. Intakes of CS+ and CS− flavor solutions were recorded (36).

Exp 4: Effects of a meal on HPV and VC GLP-1

Rats (BW 379 $\pm$ 6 g) were food deprived 1 hour before dark onset. Three hours after dark onset, the ports of both catheters were connected to polyethylene tubing (0.58 mm ID; 0.91 mm OD; Portex) the lower parts of which were sheathed with stainless-steel springs. Four hours after dark onset, a baseline blood sample (0 minutes) was taken, and rats received a 3-g chow

pellet. Additional blood samples were taken 3, 6, 10, 15, and 20 minutes after meal onset. At each time point, blood was withdrawn simultaneously from the HPV and the VC (330 μL each) into syringes containing 5 μL DPP-IV inhibitor (Millipore Corp) and 11 μL EDTA. The contents were gently mixed, transferred into an Eppendorf tube, put on ice, and centrifuged (4°C; 5000 × *g*; 8 minutes) immediately after the last blood withdrawal. Plasma (160 μL) was removed and stored at −70°C. After each of the first 4 blood withdrawals, catheters were flushed with 0.9% saline, and 330 μL donor blood was infused into the VC. After the last blood withdrawal on each experimental day, 600 μL donor blood was infused. Thereafter, the catheters were detached and food was returned.

Thirteen rats of the same strain and sex served as blood donors. Donor blood was freshly prepared each day and stored on ice until use. The donor rats were anesthetized with 5% isoflurane in oxygen. After thoracotomy the right ventricle was punctured, and two 4.5-mL blood samples were withdrawn into 5 mL-syringes containing 250 IU heparin in 0.5 mL saline, yielding a concentration of 25 IU/mL blood. Donor blood administration under comparable conditions did not affect metabolic measures or plasma corticosterone (37).

Exp 5: Effects of HPV GLP-1 infusion on VC GLP-1

Rats (BW 412 ± 7 g) were food deprived at dark onset. Two hours later the ports of both catheters were connected to polyethylene tubing as described above. Three hours after dark onset a VC baseline (0 minutes) blood sample was taken, and GLP-1 or vehicle was infused (2.5 minutes; 0.4 mL/min) into the HPV in a within-subjects cross-over design with one intervening day. Additional VC blood samples were taken at 3, 6, 10, and 25 minutes after infusion onset as described, except that no donor blood was infused. After the final blood withdrawal, the catheters were detached and food was returned.

Active GLP-1 in plasma was analyzed by ELISA (Linco Research Inc; catalog no. EGLP-35K). The intra- and interassay coefficients of variation for the GLP-1 assay were 2.6 and 3.9%, respectively. Control analyses indicated that GLP-1 values in extracted and nonextracted plasma samples were correlated with $r^2 = 0.98$.

Statistical analysis

Meals were defined as food removals more than or equal to 0.3 g lasting more than 1 minute with the interval between any two removals less than or equal to 15 minutes. The sizes and durations of the first and second meals, the duration of the first intermeal interval (IMI) (duration between first meal end and second meal onset), and cumulative food intake (CFI) at 2, 4, and 20 hours after first meal onset were analyzed with ANOVA. In Exp 1 significant main effects were followed by the Bonferroni-Holm sequential-rejection test (38). Meal pattern and CFI data from Exp 2 were analyzed with planned comparisons (Bonferroni-Holm, modified *t* tests (38)) within each surgery group and between surgery groups for the differences between GLP-1 and vehicle. In Exp 3, CS+ and CS− intakes were compared with a paired Student's *t* test. CS+ preference percentage was calculated as [Volume CS+/([Volume CS+] + [Volume CS−]) × 100 and compared between association and evocation days. Data from 3 animals that consumed less than 1 mL flavored fluid on association days were not used. Data from Exp 4 and Exp 5 were analyzed with a two-way (time × blood vessel) repeated-measures ANOVA and Bonferroni-Holm (38) planned comparisons for each time point between blood vessels and for each blood vessel between all time points and baseline. Area under the curve (AUC) was calculated relative to baseline (39) and analyzed with paired Student's *t* tests. To reduce the influence of extreme values in Exp 4 and Exp 5, data were converted to standard scores using the median absolute deviate method, and standard scores with absolute values more than 2.57 (ie, $P < .01$) were excluded. All data are expressed as means ± SEM. Differences are considered significant when $P < .05$. Statistical procedures were performed using the IBM SPSS software, version 20.0, for Exp 1–3 and SAS 0.1.3 (SAS Institute, Cary, NC) for Exp 4 and Exp 5.

Results

Exp 1: Effect of IC4V Ex-9 on the satiating effect of HPV GLP-1

Intrameal HPV GLP-1 infusion (1 nmol/kg BW) reduced the first spontaneous dark-phase meal size compared with vehicle when combined with IC4V saline infusion [GLP-1/saline vs vehicle/saline, $F(1, 7) = 11.893$, $P < .05$]. IC4V infusion of Ex-9 itself did not affect meal size (vehicle/Ex-9 vs vehicle/saline, $F(1, 7) = 0.822$, $P > .05$), but attenuated ($P < .05$) the satiating effect of HPV GLP-1 [(vehicle/Ex-9) − [GLP-1/Ex-9] vs [vehicle/saline] − [GLP-1/saline]); Bonferroni-Holm test (38) after significant ANOVA) (n = 8) (Figure 1A). First meal duration (Figure 1B), first IMI (Figure 1C), 2, 4, and 20 hours CFI (Figure 1D), and second meal size and duration (data not shown) were not affected (all $P > .05$).

Exp 2: Effect of APX on the satiating effect of HPV GLP-1

Intrameal HPV GLP-1 infusion (1 nmol/kg BW) reduced the first spontaneous dark-phase meal size ($t[6] = 2.87$; $P < .05$] (Figure 2A) and 2 hours CFI [$t(6) = 2.59$, $P < .05$] (Figure 2D) compared with vehicle in APS but not in APX rats (n = 7/group), and the differences between GLP-1 and vehicle-infused rats were significantly greater in APS than in APX rats ($t[12] = 3.59$; $P < .01$, planned comparisons (38)). First meal duration (Figure 2B), first IMI (Figure 2C), second meal size and duration (data not shown), and 4 and 20 hours CFI (Figure 2D) were not affected (all *P* values > .05) in either APS or APX rats.

Exp 3: Effect of HPV GLP-1 on flavor acceptance

Similar amounts of the orange- and lemonade-flavored solutions were consumed on association days (paired *t* test, $P > .05$), ie, neither flavor was preferred (Figure 3, A and B). On test days, CS+ (GLP-1-paired flavor) and CS− (vehicle-paired flavor) intakes were also similar ($t[15] = 0.12$, $P > .05$], and CS+ preference percentage was not

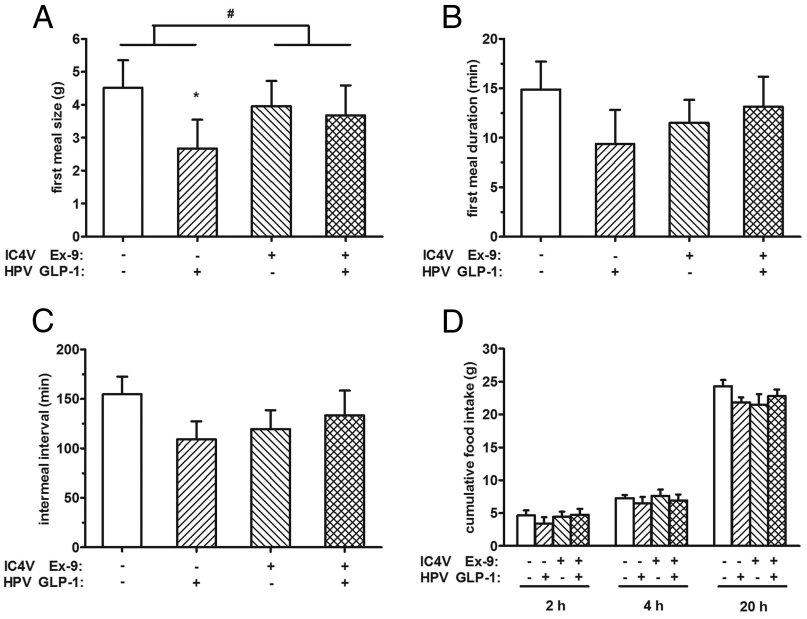

Figure 1. Effect of intrameal HPV GLP-1 (1 nmol/kg BW) infusion on meal patterns in male rats after IC4V infusion of Ex-9 (10 μg/rat) 1 hour before dark onset. Data are means ± SEM of 8 rats. *, Saline/GLP-1 smaller than saline/vehicle, $P < .05$; #, (vehicle/Ex-9 − GLP-1/Ex-9) different from (vehicle/saline − GLP-1/saline), Bonferroni-Holm sequential rejection test (38) after significant ANOVA, $P < .05$. There were no significant treatment effects (all P values > 0.05) on first meal duration (B), first intermeal interval (C), and cumulative food intake at 2, 4, and 20 hours (D).

different (t [15] = 0.73, $P > .05$) from that on association days, ie, GLP-1 did not condition flavor avoidance (Figure 3, C and D).

Exp 4: Effects of a meal on HPV and VC GLP-1

Eleven rats ate their 3-g meals in 5–6 minutes. Plasma GLP-1 concentrations changed in response to the meal (time effect: F (5, 60) = 4.02, $P < .01$) and were generally higher in the HPV than in the VC (blood-vessel effect: F (1, 56) = 12.58; $P < .01$), with a significant interaction between time and blood vessel (F (5, 96) = 3.34; $P < .05$, Figure 4). The planned comparisons revealed that HPV GLP-1 increased between 3 and 6 minutes after meal onset (3 minutes vs 0 minutes: $P > .05$; 6 minutes vs 0 minutes: $P < .01$) and returned to baseline between 15 and 20 minutes after meal onset (20 minutes vs 0 minutes: $P > .05$). No significant changes in VC GLP-1 concentrations were detected. HPV GLP-1 concentrations were higher than VC concentrations at 6 and 15 minutes after meal onset (HPV vs VC: $P < .01$), and the AUC for GLP-1 was greater in the HPV than in the VC (t [8] = 2.81, $P < .05$, Figure 4).

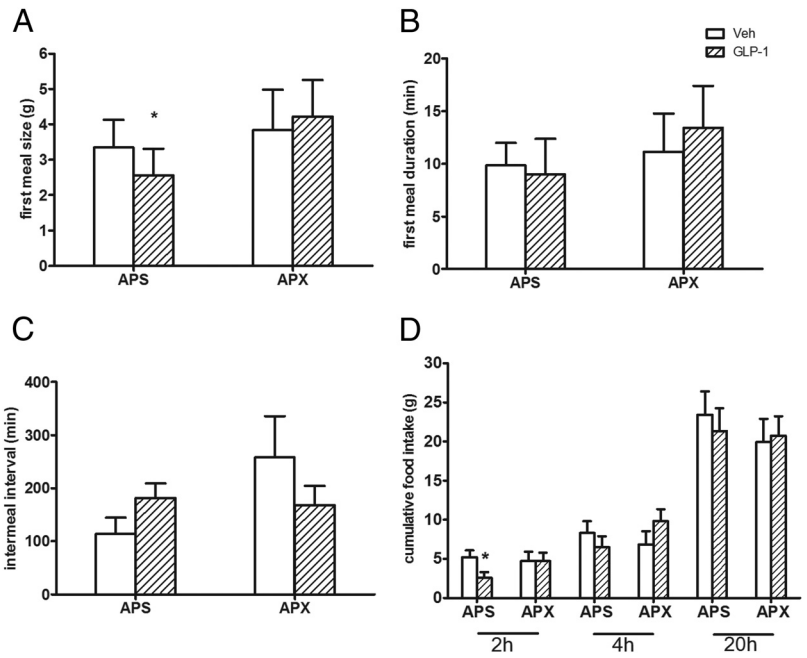

Figure 2. Effect of intrameal HPV GLP-1 (1 nmol/kg BW) infusion on meal patterns in male rats after APX (n = 7) or sham surgery (APS) (n = 7). Data are means ± SEM. *, HPV GLP-1 reduced ($P < .05$) first meal size (A) and 2 hours cumulative food intake (D) in sham-operated rats, but not ($P > .05$) in APX rats. There were no significant treatment effects ($P > .05$) on first meal duration (B), first intermeal interval (C), and cumulative food intake at 4 and 20 hours (D) in both surgical groups. Veh, vehicle.

Exp 5: Effects of HPV GLP-1 infusion on VC GLP-1

HPV GLP-1 infusion (1 nmol/kg BW) increased (F (4, 45) = 19.57, $P < .001$) VC plasma concentrations of active GLP-1 3 minutes after infusion onset (3 vs 0 minutes: $P < .01$; 3 minutes GLP-1 vs vehicle: $P < .01$), but no significant differences were detected later (all $P > .05$; Figure 5). VC GLP-1 concentrations did not change over time in vehicle-treated rats. The AUC for GLP-1 was greater in GLP-1 than in vehicle-treated rats (t [5] = 3.66; $P < .01$, Figure 5).

Discussion

Our results 1) implicate AP and hindbrain GLP-1R in the eating-inhibitory effect of HPV infused GLP-1; 2) indicate that circulating GLP-1 can inhibit eating without inducing fla-

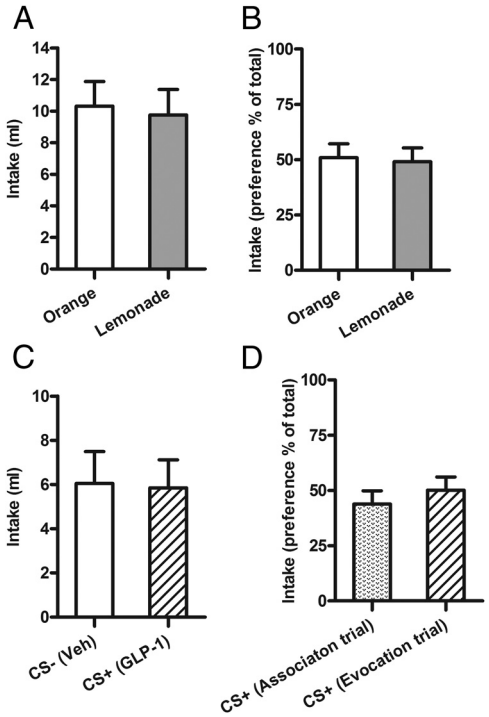

Figure 3. Effect of HPV GLP-1 (1 nmol/kg BW) infusion on conditioned flavor acceptance in 16 rats. Equal consumption of orange- and lemon-flavored saccharin solutions on association days (A and B). Similar intake of a CS+ (GLP-1) and CS− (Vehicle) solution on evocation day (C) and of CS+ on evocation day as compared with the association days (D). Intake values are shown in milliliters and preference percentage of total intake as mean ± SEM, paired Student's *t* test, $P > .05$.Veh, vehicle.

vor avoidance; and 3) reveal that a chow meal in briefly food-deprived rats acutely and transiently increased the plasma concentration of active GLP-1 in the HPV, but not systemically (VC). Finally, 4) HPV infusion of GLP-1 (1 nmol/kg BW) produced a transient, but supraphysiological increase in systemic plasma concentration of active

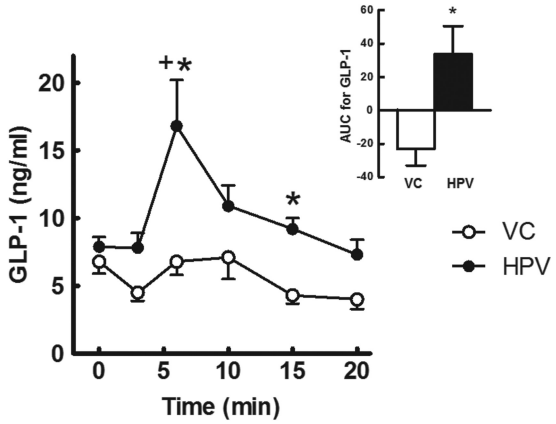

Figure 4. Effects of a 3-g chow meal in rats on plasma levels of active GLP-1, as well as on the AUC (inset), in the HPV and the VC. The meal was given 4 hours into the dark phase after 5 hours of food deprivation. Data are means ± SEM of 11 rats. +, $P < .01$ vs baseline (0 minutes); *, $P < .01$ HPV vs VC.

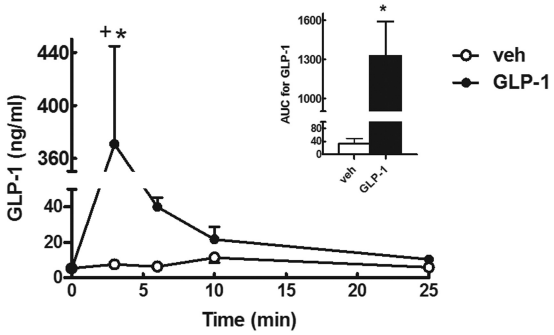

Figure 5. Effects of a HPV infusion (2.5 minutes, 0.4 mL/min) of GLP-1 (1 nmol/kg BW) on plasma levels of active GLP-1, as well as on the AUC (inset), in the VC. The infusion was given 3 hours into the dark phase after 5 hours of food deprivation. Data are means ± SEM of 12 rats. +, $P < .01$ vs baseline (0 minutes); *, $P < .01$ vs vehicle (veh).

GLP-1. Thus, satiation in response to HPV GLP-1 infusion may reflect a pharmacologic rather than a physiological effect under our conditions. Likewise, activation of hindbrain GLP-1R by circulating endogenous GLP-1 is probably of limited importance for normal satiation in this particular situation. Nevertheless, activation of hindbrain GLP-1R may be relevant when circulating GLP-1 or GLP-1R agonists are substantially increased, as may occur during other types of meals or in patients after bariatric surgery or pharmacologic treatments.

IC4V infusion of the GLP-1R antagonist Ex-9 blocked the satiating effect of an intrameal HPV GLP-1 infusion, but did not increase meal size by itself. This indicates that hindbrain GLP-1R that are accessible from the fourth ventricle, presumably located in the AP, mediate the acute eating-inhibitory effect of circulating exogenous GLP-1. The findings also indicate that activation of these receptors is not required for normal satiation under our conditions. Our findings are consistent with previous reports (40) showing that chow intake did not increase with IC4V Ex-9 infusion in overnight food-deprived rats in the absence of a prior nutrient preload. Hindbrain GLP-1R blockade did, however, antagonize the satiation induced by artificial gastric distension or consumption of a nutrient preload, although not satiation induced by intraduodenal nutrient delivery. Therefore, the ingestion of a large meal and HPV GLP-1 infusion may activate hindbrain GLP-1R and similar brain circuitries to inhibit eating.

The stronger effects of intrameal HPV GLP-1 infusion on first meal size and 2-hour CFI in APS than in APX in Exp 2 indicate that an intact AP is required for the full expression of the eating-inhibitory effect of circulating GLP-1. The AP with its leaky blood brain barrier is accessible to circulating GLP-1 (18, 19). In line with this, iv injection of protease-resistant albumin-conjugated GLP-1, which cannot cross the blood brain barrier, induced an increase in AP c-Fos expression (41), similar to

HPV GLP-1 infusion in our hands (19). Remotely controlled intrameal HPV GLP-1 infusions previously reduced the size of the ongoing meal (15, 19) without affecting the subsequent IMI. This is consistent with the short biological half-life of GLP-1, which was also observed in Exp 5. GLP-1 reduced first nocturnal meal size in Exp 1 and Exp 2, although the effect was less reliable in Exp 2, in which the APS rats displayed a great between-subject variability. Such variability in peptide effects on eating is a frequently encountered phenomenon (42). The SEM, of course, also is increased by consistent within-subjects variability, whereas the SE of the difference (SED) is not. The statistical comparisons depend on the SED, not the SEM. The SED of first meal size difference was 0.27 g, which was small in relation to the difference between means (0.79 g). Despite the small difference in first meal size, HPV GLP-1 infusion clearly reduced 2 hours CFI in APS rats because not all animals started a second meal within this time.

Intraperitoneal injection of Ex-4 (2.0 μg/kg BW) was reported to reduce food intake up to 2 hours in APX rats after 20 hours of food deprivation (43). This seems to contradict the idea that the AP mediates the eating-inhibitory effect of circulating GLP-1. In that study, however, Ex-4 was administered ip, and we showed that abdominal vagal afferents are necessary for the early satiating effect of ip Ex-4 (44). It is therefore possible that the initial Ex-4 effect in that study was also mediated by vagal afferents, independent of the AP. Further experiments should examine whether the eating-inhibitory effect of circulating GLP-1 and GLP-1R agonists are mediated by GLP-1R-expressing neurons in the AP.

Our findings are consistent with the hypothesis that peripherally administered GLP-1 can inhibit eating through activation of 2 partially separate pathways: ip administered GLP-1 through a paracrine action on primarily intestinal vagal afferents (15, 45), and iv infused GLP-1 through an endocrine action on hindbrain GLP-1R. Further support for central and peripheral sites of action of GLP-1 is derived from the finding that IC3V Ex-9 administration blocked the eating inhibition by IC3V infusion (46, 47), but not ip injection, of GLP-1 (47). The AP transduces humoral interoceptive information (48) and can elicit rapid homeostatic responses to fluid and nutrient imbalances (18). Intravenously, but not intra-cerebroventricularly, administered GLP-1R agonists induced tyrosine hydroxylase transcription in the AP (49), and AP GLP-1-responsive tyrosine hydroxylase neurons project to various sites, including the NTS (41). In chronic supracollicular decerebrate rats, hindbrain processing of the signal derived from hindbrain GLP-1R activation was sufficient to inhibit eating (50). We do not know, however, whether this is also the case when circulating GLP-1 activates hindbrain GLP-1R. The hypothalamic Arc and PVN seem to mediate the effects of centrally administered GLP-1 on glucose metabolism and eating, respectively (51). After HPV GLP-1 infusion we observed increased c-Fos expression in the NTS and AP, but not in the PVN and Arc (19). NTS GLP-1 neurons project to many brain areas (52) including the PVN (53), and they do not express GLP-1R (54). It is therefore likely that HPV-infused GLP-1 induces satiation by activating a hindbrain circuit that does not involve NTS GLP-1 neurons or by activating hindbrain projections to brain areas other than the Arc and PVN.

Exp 3 did not reveal a flavor-avoidance reaction after HPV GLP-1 infusion, indicating that satiation after HPV GLP-1 infusion (15, 19) can be dissociated from flavor avoidance. The 2-bottle conditioned flavor avoidance protocol that we used permits comparison of the consumption of 2 similar solutions in a within subjects cross-over design and is considered very sensitive (36). If GLP-1 did induce malaise under our conditions, the animals should have avoided the flavor associated with it (ie, CS+) and preferentially consumed the "safe" flavor (ie, CS−) (44). Because this was not the case, our behavioral findings suggest that the inhibition of eating by HPV GLP-1 reflects satiation rather than malaise. This method has similarly dissociated satiation and flavor avoidance produced by peptide tyrosine-tyrosine (26), Ex-4 (44), and diacylglycerol acyltransferase-1 inhibitor (55). The amygdala has been implicated in the learned avoidance induced by central GLP-1 administration (23–25). Kinzig et al (25) demonstrated that intraamygdala administration of GLP-1 produced a strong conditioned taste aversion without affecting food intake and that intraamygdala administration of Ex-9 prevented taste aversion learning in response to ip LiCl injections. The CeA is generally known to process hedonic information. Our previous finding of increased CeA c-Fos expression after HPV GLP-1 infusion suggests that some change in hedonics occurs (19). If it was a negative change, it was apparently too weak to be detected by the sensitive behavioral test procedure employed here and therefore was probably not a major contributor to the inhibition of eating. Whether HPV GLP-1 infusion activates GLP-1R-expressing neurons in the CeA deserves further study.

Previous reports of plasma GLP-1 levels in rats often involved intragastric (3, 56) or intraintestinal (57, 58) administrations of liquid diets (3, 57) or nutrient solutions (56–58) in anesthetized animals, ie, situations that do not closely mimic a solid, mixed-nutrient meal in awake, behaving animals. In humans, meal-related changes in circulating GLP-1 have been reported (59–62), but these

studies did not measure GLP-1 in the HPV. We measured active GLP-1 because circulating GLP-1 is quickly degraded by DPP-IV (59, 63, 64). Much of circulating total GLP-1 in humans is deactivated GLP-1 (65), which is rapidly eliminated by renal filtration (5, 64). Thus, total GLP-1 reflects secreted hormone, but is not a useful measure of the active circulating hormone. Active HPV GLP-1 levels peaked 6 minutes after meal onset, but VC GLP-1 levels did not increase. Further, HPV GLP-1 infusion increased VC GLP-1 3 minutes after infusion onset much more than the increase in active HPV GLP-1 during a meal. Thus, HPV-infused GLP-1 produced systemic increases in GLP-1 that were sufficient to elicit a central effect, but the lack of a systemic increase in endogenous GLP-1 during a real, mixed-nutrient meal of similar size questions the physiological relevance of the satiating effect of HPV-infused GLP-1 under our conditions.

A substantial postprandial increase in circulating endogenous GLP-1 may contribute to the antidiabetic and antiobesity effects of bariatric surgery (66). Also, chronic administration of GLP-1R agonists reduces BW in obese patients (67), and GLP-1 enhances glucose-stimulated insulin release and insulin sensitivity (68, 69). Together, this supports a promising role of GLP-1-based therapies for the treatment of obesity and type 2 diabetes mellitus (70).

In sum, we previously showed that the eating-inhibitory effect of circulating GLP-1 is not dependent on vagal afferents and is associated with neuronal activation in the NTS and AP (15, 19). We here extend these findings by demonstrating that the eating-inhibitory effect of HPV GLP-1 infusion under our conditions 1) involves hindbrain GLP-1R activation, 2) requires an intact AP, and 3) can be dissociated from malaise. Although the exact central neurochemical and intracellular signaling mechanisms that lead to the eating-inhibitory effect of circulating GLP-1 remain uncertain, the present findings expand our knowledge about these mechanisms and their potential clinical implications.

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