Duodenal Activation of cAMP-Dependent Protein Kinase Induces Vagal Afferent Firing and Lowers Glucose Production in Rats

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BACKGROUND & AIMS: The duodenum senses nutrients to maintain energy and glucose homeostasis, but little is known about the signaling and neuronal mechanisms involved. We tested whether duodenal activation of adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase A (PKA) is sufficient and necessary for cholecystokinin (CCK) signaling to trigger vagal afferent firing and regulate glucose production. **METHODS:** In rats, we selectively activated duodenal PKA and evaluated changes in glucose kinetics during the pancreatic (basal insulin) pancreatic clamps and vagal afferent firing. The requirement of duodenal PKA signaling in glucose regulation was evaluated by inhibiting duodenal activation of PKA in the presence of infusion of the intraduodenal PKA agonist (Sp-cAMPS) or CCK1 receptor agonist (CCK-8). We also assessed the involvement of a neuronal network and the metabolic impact of duodenal PKA activation in rats placed on high-fat diets. **RESULTS:** Intraduodenal infusion of Sp-cAMPS activated duodenal PKA and lowered glucose production, in association with increased vagal afferent firing in control rats. The metabolic and neuronal effects of duodenal Sp-cAMPS were negated by coinfusion with either the PKA inhibitor H89 or Rp-CAMPS. The metabolic effect was also negated by coinfusion with tetracaine, molecular and pharmacologic inhibition of NR1-containing *N*-methyl-D-aspartate (NMDA) receptors within the dorsal vagal complex, or hepatic vagotomy in rats. Inhibition of duodenal PKA blocked the ability of duodenal CCK-8 to reduce glucose production in control rats, whereas duodenal Sp-cAMPS bypassed duodenal CCK resistance and activated duodenal PKA and lowered glucose production in rats on high-fat diets. **CONCLUSIONS: We identified a neural glucoregulatory function of duodenal PKA signaling.**

Keywords: Intestine; Animal Model; Nervous System; Diabetes.

BASIC AND TRANSLATIONAL AT

Approximately 220 million people have type 2 diabetes, and almost half of this population lives in China.[1,2] Diabetes and obesity are characterized by a disruption in the homeostatic control of glucose and energy homeostasis. The disruption is caused in part by an elevation of hepatic glucose production and appetite, respectively. Studies aimed to unveil novel signaling molecules that lower glucose production and food intake are important in advancing the search for effective antidiabetic and antiobesity therapies.

The duodenum detects a rise in nutrients to ignite negative feedback systems to maintain peripheral homeostasis.[3] Activation of biochemical pathways that metabolize preabsorptive lipids within the duodenum concurrently inhibits glucose production and food intake.[3–5] The underlying mechanisms remain elusive but the secretion of cholecystokinin (CCK) from the duodenal I cells and the binding to its gut CCK1 receptors (CCK1 was formerly referred to as CCK-A) are sufficient and necessary for lipids to trigger in parallel a gut-brain and a gut-brain-liver axis to lower appetite[6–10] and glucose production,[11] respectively. In addition, CCK alters digestion and improves nutrient absorption by stimulating pancreatic amylase secretion, promotes bile release from the gallbladder, and delays gastric emptying.[12] Importantly, the physiological relevance of duodenal CCK action in glucose regulation is shown by the fact that molecular and pharmacologic inhibition of duodenal CCK1 receptor disrupts glucose homeostasis during refeeding.[11]

The CCK1 receptor is mostly expressed in the gut, and its signaling pathways have been studied in the pancreatic acinar cell.[13] The CCK1 receptor belongs to the family of G protein–coupled receptors.[14] The classic G protein–coupled receptor signaling pathways involve both protein kinase A (PKA) and phospholipase C activation, and both signaling pathways have been described to mediate the direct CCK/CCK1 receptor signaling cascade in pancreatic secretions.[13,15–17] To date, it is unknown whether G protein–coupled signaling pathway(s) via PKA mediate CCK1 receptor signaling in duodenal vagal afferent terminals to regulate peripheral glucose homeostasis, and this working hypothesis is tested in the current study.

Given that duodenal CCK resistance is acquired in response to high-fat feeding,[5–7,11] studies aimed to dissect the duodenal CCK1 receptor signaling cascade (ie, PKA signaling) that regulates glucose production in normal and high-fat fed rats will begin locating the molecular defects in duodenal CCK resistance. Consequently, novel signaling molecules within the duodenum could

Abbreviations used in this paper: DVC, dorsal vagal complex; NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; PLC, phospholipase C.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.12.053

be targeted to restore glucose homeostasis in diabetes and obesity.

In the current study, we propose that direct activation of the duodenal PKA signaling pathway is necessary for the CCK/CCK1 receptor and sufficient to trigger vagal afferent firing to activate a neuronal network to lower glucose production in rats in vivo.

Materials and Methods

Animal Preparation

The animal study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of University Health Network. Adult 8-week-old male Sprague–Dawley rats (280–300 g) were obtained from Charles River Laboratories (Montreal, Quebec, Canada). Rats were housed individually, were maintained on a standard light-dark cycle, and had ad libitum access to rat chow and water. Three to 4 days before the in vivo clamp studies, rats were subjected to duodenal cannulations to insert a catheter into the duodenum approximately 2 cm downstream of the pyloric sphincter and cannulations in the internal jugular vein and carotid artery for infusion and blood sampling.[4,11] A group of rats also received hepatic vagotomy. Another group of rats underwent stereotaxic surgery for implantation of a bilateral catheter targeting the nucleus of solitary tract within the dorsal vagal complex (DVC)[4,11] 5 days before vascular and duodenal cannulations. The stereotaxic coordinates used were 0.0 mm on the occipital crest, 0.4 mm lateral to the midline for both sides, and 7.9 mm below the skull surface. Recovery from surgery was monitored daily by measuring daily food intake and weight gain 3–4 days after surgery.

Electrophysiological Ex Vivo Recordings of Duodenum Preparation

The duodenum (just below the sphincter of Oddi, ~5 cm long) was removed from anesthetized (80 mg/kg pentobarbital intraperitoneally) adult male Sprague–Dawley rats (200–250 g) and placed immediately in a recording chamber and subsequently perfused with oxygenated Krebs' solution as previously described.[18,19] Both ends of the duodenum segment were cannulated to allow intraluminal infusion of solutions and ramp distentions (9 mL/h, up to 60 mm Hg) at 15-minute intervals. A branch of the mesenteric nerves, consisting of both vagal and spinal afferents, was dissected and recorded using a suction electrode. After the spontaneous afferent nerve discharge and the distention-induced activity became stable, the intraluminal infusion solution was switched to 30 μmol/L Sp-cAMPS (in Krebs' solution, 9 mL/h for 30 minutes; Tocris Bioscience, Ellisville, MO). In a separate set of experiments, intraduodenal infusion of H-89 (12 μmol/L) was coinfused with Sp-cAMPS.

Pancreatic Euglycemic Clamp Procedure

The clamp was performed as described.[4,11] Rats were restricted to ~56 kcal of caloric intake the night before the experiments. A primed continuous infusion of [3-^{3}H]-glucose (PerkinElmer, Woodbridge, ON, Canada; 40 μCi bolus; 0.4 μCi/min) was given from the start of the experiment (t = 0 minutes) to the end (t = 200 minutes) to assess glucose kinetics based on tracer-dilution methodology. From t = 90 to t = 200 minutes, insulin (1.2 $mU \cdot kg^{-1} \cdot min^{-1}$) and somatostatin (3 $\mu g \cdot kg^{-1} \cdot min^{-1}$) were infused. A variable infusion of 25% glucose solution was started at t = 90 minutes and periodically adjusted every 10 minutes until t = 200 minutes. When required, MK-801 (0.03 ng/min; Sigma) was administered into the DVC from t = 90 to t = 200 minutes. Intraduodenal infusions were performed from t = 150 to t = 200 minutes to determine the effects of different duodenal treatments on glucose kinetics. To determine the specific activity of [3-^{3}H]-glucose and insulin levels, plasma samples were taken at 10-minute intervals. At the end of the experiment, rats were killed and tissue samples were freeze clamped in situ with steel tongs precooled in liquid nitrogen. Tissues were stored at −80°C until use.

Intraduodenal Infusions and Treatments

The following substances were infused into the duodenum at a rate of 0.01 ml/min: (1) saline, (2) Sp-cAMPS (30 μmol/L), (3) H-89 (12 μmol/L; Tocris Bioscience), (4) Rp-CAMPS (12 μmol/L; Tocris Bioscience), (5) tetracaine anesthetic (0.015 mg/min; Sigma, St. Louis, MO), and (6) CCK-8 (35 $pmol \cdot kg^{-1} \cdot min^{-1}$; Sigma). Of note, a same set of Sp-cAMPS infusion clamp experiments was performed but with intrajejunal (instead of intraduodenal) Sp-cAMPS infusion. Intrajejunal Sp-cAMPS did not inhibit glucose production (12.2 ± 2.0 $mg \cdot kg^{-1} \cdot min^{-1}$; n = 5).

High-Fat Diet Feeding

Rats were placed on a lard oil–enriched diet (Supplementary Table 4) after duodenal and intravenous catheter implantation for 3 days. Rats develop defects in duodenal lipid-CCK sensing mechanisms.[4,11]

PKA Activity Assay

PKA activity in duodenal samples, taken directly after the clamp studies, was measured with the PepTag Assay Kit (Promega, Madison, WI) with minor modifications. Briefly, 1 g of duodenal tissue was homogenized in ice-cold PKA extraction buffer containing 25 mmol/L Tris-HCl (pH 7.4), 0.5 mmol/L EDTA & EGTA, 0.5 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 10 mmol/L β-mercaptoethanol, and 3× Complete Mini EDTA-Free Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Laval, QC, Canada). The homogenates were centrifuged at 12,300 rpm for 5 minutes at 4°C. An equal amount of protein was allowed to react with the PepTag A1 peptide for 30 minutes at 37°C. The reaction was stopped with incubation at 95°C. Samples were run on a 0.8% agarose gel at 100 V for 15 minutes. Data were analyzed using ImageJ (National Institutes of Health software).

Adenoviral Infection in the DVC

Immediately after the stereotaxic surgery, rats received 3 μL of adenovirus (adenovirus containing short hairpin RNA-NR1: 4.0×10^{11} plaque-forming units/mL; mismatch, 4.0×10^{11} plaque-forming units/mL) per side of the cannulae over a 30-second injection. Microsyringes were left in the cannula for 20 minutes before removal to prevent any backflow. We verified that direct injection of this adenovirus into the DVC knocked down the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptors.[20,21]

Hepatic Branch Vagotomy

The hepatic vagus of the ventral subdiaphragmatic vagal trunk was transected by microcautery, disrupting the neural communications between the liver and the brain as well as a small portion of the intestinal nerve supply.[4,11]