Duodenal Mucosal Protein Kinase C-δ Regulates Glucose Production in Rats

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BACKGROUND & AIMS: Activation of protein kinase C (PKC) enzymes in liver and brain alters hepatic glucose metabolism, but little is known about their role in glucose regulation in the gastrointestinal tract. We investigated whether activation of PKC-δ in the duodenum is sufficient and necessary for duodenal nutrient sensing and regulates hepatic glucose production through a neuronal network in rats. **METHODS:** In rats, we inhibited duodenal PKC and evaluated whether nutrient-sensing mechanisms, activated by refeeding, have disruptions in glucose regulation. We then performed gain- and loss-of-function pharmacologic and molecular experiments to target duodenal PKC-δ; we evaluated the impact on glucose production regulation during the pancreatic clamping, while basal levels of insulin were maintained. **RESULTS:** PKC-δ was detected in the mucosal layer of the duodenum; intraduodenal infusion of PKC inhibitors disrupted glucose homeostasis during refeeding, indicating that duodenal activation of PKC-δ is necessary and sufficient to regulate glucose homeostasis. Intraduodenal infusion of the PKC activator 1-oleoyl-2-acetyl-sn-glycerol (OAG) specifically activated duodenal mucosal PKC-δ and a gut-brain-liver neuronal pathway to reduce glucose production. Molecular and pharmacologic inhibition of duodenal mucosal PKC-δ negated the ability of duodenal OAG and lipids to reduce glucose production. **CONCLUSIONS: In the duodenal mucosa, PKC-δ regulates glucose homeostasis.**

Keywords: Intestine; Animal Model; Signaling; Diabetes.

The various protein kinase C (PKC) isoforms are involved in an immense array of biologic functions. In particular, the PKC isoform PKC-δ is activated by the accumulation of lipid-derived metabolites such as long-chain fatty acyl-CoA (LCFA-CoA) or diacylglycerol in the liver to alter the regulation of hepatic glucose production by insulin.[1–3] On the other hand, short-term accumulation of lipid-derived metabolites such as LCFA-CoA in the mediobasal hypothalamus lowers hepatic glucose production,[4,5] in association with an activation of hypothalamic PKC-δ.[6] These studies in parallel highlight the importance of PKC-δ in mediating lipid sensing to regulate glucose homeostasis in vivo.

In addition to metabolic effects in the liver and the brain, a short-term accumulation of LCFA-CoA in the duodenum lowers glucose production through a gut-brain-liver neuronal axis.[7] Although cholecystokinin (CCK) action in the intestine is required for intestinal lipid metabolism to lower glucose production,[8] the downstream effectors of intestinal lipid and/or CCK action remain unknown. Interestingly, activation of PKC has been reported to mediate the ability of fatty acids to stimulate the release of CCK in CCK-secreting cell line (STC-1),[9,10] and this finding raises the possibility that duodenal PKC mediates lipid sensing to regulate glucose production and homeostasis in vivo.

Using normal male Sprague–Dawley rats, we first tested whether direct pharmacologic inhibition of duodenal PKC disrupts nutrient-sensing mechanisms activated by refeeding to regulate glucose homeostasis. In an intestinal-specific manner, we then introduced bidirectional changes in duodenal mucosal PKC-δ activity with molecular and pharmacologic approaches and evaluated whether activation of duodenal PKC-δ is sufficient and necessary for lipid sensing to regulate glucose production in vivo (Supplementary Figure 1).

Materials and Methods

Animal Preparation

The animal study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University Health Network. Nine-week-old male Sprague–Dawley rats (250–300 g; Charles River Laboratories, Montreal, QC, Canada) were used for all experiments. Rats were housed in individual cages and were maintained on a standard light-dark cycle. The animals had access to rat chow and water ad libitum and were given 7 days to acclimatize before the designated surgeries were performed.

Duodenal and Intravenous Cannulations and Stereotaxic Surgery

Duodenal cannulation surgeries were performed to insert a catheter into the proximal duodenum (approximately 1.5–2 cm downstream of the pyloric sphincter). Intravenous

Abbreviations used in this paper: AD, adenovirus; BIM, bisindolylmaleimide; DMSO, dimethyl sulfoxide; DN, dominant negative; LCFA-CoA, long-chain fatty acyl-CoA; NMDA, *N*-methyl-D-aspartate; NTS, nucleus of the solitary tract; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PKC, protein kinase C.

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catheters were placed into the internal jugular vein and carotid artery for the purpose of infusion and blood sampling, respectively, during the clamp protocol. These surgical procedures were performed as previously described.[7,8] Bilateral catheters were inserted into the dorsal vagal complex targeting the nucleus of the solitary tract (NTS) into a subgroup of rats. The stereotaxic brain surgeries were performed 7 days before the duodenal and intravenous cannulations were inserted, allowing ample recovery time. The coordinates were 0.0 mm on the occipital crest, 0.8 mm (left and right) lateral to the midline, and 7.9 mm below the skull surface. Animals were monitored for daily food intake and body weight for 3 to 4 days after surgery, and these parameters were used as outcome measures for surgical recovery.

Intraduodenal Infusion and the Pancreatic (Basal Insulin)-Euglycemic Clamp Procedure

The night before the pancreatic clamp procedure took place, rats were restricted to approximately 58 kcal of food intake to ensure the same nutritional postabsorptive status at the time of the experiment. At t = 0 minutes, a primed continuous infusion of [3-^{3}H]-glucose (bolus, 40 μCi; 0.4 μCi/min) was given and this rate was maintained throughout the remainder of the experiment to assess changes in the rate of glucose production and whole body glucose uptake using tracer-dilution methodology. The pancreatic clamp was started 90 minutes after the initiation of the tracer infusion (t = 90 minutes) to allow adequate time for the tracer to reach steady state. At this time, a continuous infusion of insulin at 0.8 mU $\times$ kg^{-1} $\times$ min^{-1} and somatostatin (inhibitor of endogenous insulin and glucagon secretion) at 3 μg $\times$ kg^1 $\times$ min^{-1} was initiated. A 25% glucose solution was infused at a variable rate to maintain euglycemia (euglycemia was determined during basal levels from t = 60 minutes to t = 90 minutes). The exogenous glucose infusion rate was adjusted every 10 minutes from t = 120 to 200 minutes. Intraduodenal infusions were initiated at t = 150 minutes and maintained until t = 200 minutes (0.01 mL/min). In a subgroup of rats undergoing NTS treatment procedures, MK801 was initiated at t = 90 minutes and maintained until the completion of the experiment. Additional blood samples were taken at t = 90, 180, and 200 minutes to determine plasma insulin levels.

The following chemicals were used where necessary for the experiments: (1) saline; (2) 1-oleoyl-2-acetyl-sn-glycerol (OAG): PKC activator (Calbiochem, San Diego, CA; stock solution made in dimethyl sulfoxide [DMSO], working solution dissolved in saline; 250 μmol/L [0.4% final concentration of DMSO]); (3) bisindolymaleimide (BIM): general PKC inhibitor (Tocris Bioscience, Ellisville, MO; stock solution made in DMSO, working solution dissolved in saline; 240 μmol/L [1.3% final concentration of DMSO]); (4) rottlerin: PKC-δ specific inhibitor (Calbiochem; stock solution made in DMSO, working solution dissolved in saline; 60 μmol/L [3.0% final concentration of DMSO]); (5) tetracaine: general anesthetic (Sigma, St. Louis, MO; stock solution made in DMSO, working solution dissolved in saline; 1 mg/mL [0.5% final concentration of DMSO]); (6) MK801: *N*-methyl-D-aspartate receptor inhibitor (Sigma; stock solution made in saline, working solution dissolved in saline; 5 μg/mL at 0.03 ng/min) (MK-801, when administered into the NTS alone at this rate, does not alter glucose kinetics during the clamps[7] but at a much higher dose as a single bolus would induce sucrose drinking[11]); (7) 20% Intralipid (Baxter Corporation; 0.03 kcal/min): the intraduodenal infusions were infused during the pancreatic clamp from t = 150 to 200 minutes at a rate of 0.01 mL/min.

Fasting-Refeeding Protocol

Fasting-refeeding experiments were performed in a subgroup of rats that had undergone duodenal cannulation. Animals were fasted beginning at 4 PM the day before the onset of the experiment; the experiments were initiated at exactly 4 PM the following day, allowing for a complete 24-hour fast. At 4 PM, a 10-minute preinfusion of the general PKC inhibitor BIM (240 μmol/L) or saline (control) was administered (0.01 mL/min), which was maintained for 20 minutes following the preinfusion. Regular chow was given back to the animals at t = 0 minutes. Food intake and glucose levels were measured every 10 minutes (ie, at t = $-$10, 0, 10, 20 minutes).

Virus Injection and Surgery

Proximal duodenal segment (~4 to 5 cm) was isolated after a midline laparotomy. About 1 to 1.5 cm downstream from the pyloric sphincter, 2 ligatures of 2 cm apart were made around the duodenum using silk sutures to restrict outward flow of virus and inward flow of intestinal fluids. A hole was punctured in the middle of the 2 ligatures with a 23-gauge needle. The intestinal contents were gently massaged, and the lumen of this region was flushed with 0.2 to 0.5 mL of warmed saline. The saline was then milked from the segment, and 0.2 mL of adenovirus expressing the LacZ or the dominant negative form (DN) of PKC-δ (4×10^8 pfu/mL) (gift from Dr J. Soh, Korea) was injected into the lumen. The injection site was marked with small suture knots to prevent outflow of the virus. The duodenal segment was returned back into the abdominal cavity and kept moist for 20 minutes to enhance virus binding and uptake. After 20 minutes, the 2 ligatures at both ends of the segment, as well as the knots on top of the injection site, were removed. A duodenal catheter was subsequently inserted through the injection site, followed by the vascular cannulation in the same procedures as described earlier. Animals were allowed to recover for 3 days before the clamp procedures.

Western Blotting for PKC

Duodenal mucosal tissue was taken immediately after the animal was killed. The tissue was rinsed in phosphate-buffered saline, and mucosal tissue was scraped on ice. The muscle layer was discarded, and mucosal tissue was placed in solution containing 150 μL buffer A (50 mmol/L Tris-HCl, pH 7.5, 10 mmol/L ethylene glycol tetraacetic acid, 2 mmol/L EDTA, 1 mmol/L $NaHCO_3$, 5 mmol/L $MgCl_2$, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, 1 μg/mL aprotinin, leupeptin, pepstatin, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 μmol/L microcystin) and stored at $-$80°C. A total of 50 μL of homogenized duodenum containing buffer A was spun at 1 hour at 100,000*g* at 4°C. The supernatant (cytosolic fraction) was obtained. A total of 50 μL buffer B (buffer A containing Triton X-100) was added to the remaining pellet fraction and resuspended. The resuspended sample was kept on ice for 30 minutes and then respun for 1 hour at 100,000*g* at 4°C. The supernatant was obtained (membrane fraction). Western blotting analysis was performed on PKC-δ (1:50) isoform using antibodies from Santa Cruz Biotechnology (sc-213; Santa Cruz, CA). Western blotting analysis was also performed for PKC-ε (antibodies from Santa Cruz Biotechnology), but the PKC-ε was undetectable in the duodenal mucosa as previously reported by another study.[12]

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