

# Duodenal PKC- $\delta$ and Cholecystokinin Signaling Axis Regulates Glucose Production

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**OBJECTIVE**—Metabolism of long-chain fatty acids within the duodenum leads to the activation of duodenal mucosal protein kinase C (PKC)- $\delta$  and the cholecystokinin (CCK)-A receptor to lower glucose production through a neuronal network. However, the interfunctional relationship between duodenal PKC- $\delta$  and CCK remains elusive. Although long-chain fatty acids activate PKC to stimulate the release of CCK in CCK-secreting cells, CCK has also been found to activate PKC- $\delta$  in pancreatic acinar cells. We here evaluate whether activation of duodenal mucosal PKC- $\delta$  lies upstream (and/or downstream) of CCK signaling to lower glucose production.

**RESEARCH DESIGN AND METHODS**—We first determined with immunofluorescence whether PKC- $\delta$  and CCK were colocalized within the duodenal mucosa. We then performed gain- and loss-of-function experiments targeting duodenal PKC- $\delta$  and the CCK-A receptor and evaluated the impact on changes in glucose kinetics during pancreatic (basal insulin) clamps in rats *in vivo*.

**RESULTS**—Immunostaining of PKC- $\delta$  was found to colocalize with CCK in the duodenal mucosa. Intraduodenal coinfusion of either the CCK-A receptor antagonist MK-329 or CR-1409 with the PKC activator negated the ability of duodenal mucosal PKC- $\delta$  activation to lower glucose production during the pancreatic clamps in normal rats. Conversely, molecular and pharmacological inhibition of duodenal PKC- $\delta$  did not negate the ability of the duodenal CCK-A receptor agonist CCK-8 to lower glucose production, indicating that activation of duodenal PKC- $\delta$  lies upstream (and not downstream) of CCK signaling. Finally, intraduodenal PKC activator infusion failed to lower glucose production in rats with high-fat diet-induced duodenal CCK resistance.

**CONCLUSIONS**—In summary, activation of duodenal PKC- $\delta$  leads to the stimulation of CCK release and activation of the CCK-A receptor signaling axis to lower glucose production in normal rats, but fails to bypass duodenal CCK-resistance in high fat-fed rats. *Diabetes* 60:3148–3153, 2011

**L**ipid metabolism within the duodenum increases long-chain fatty acyl-CoA levels (1) and cholecystokinin (CCK) release that leads to the activation of duodenal CCK-A receptors and an inhibition of glucose production (2). The necessary step(s) that mediate

the ability of duodenal long-chain fatty acyl-CoA to stimulate CCK release to regulate glucose production, however, remains unknown.

Novel protein kinase C (PKC) isoforms such as  $\delta$  and  $\theta$  are activated by lipids in the brain (3,4) and  $\delta$ ,  $\theta$ , and  $\epsilon$  in the liver (5) to regulate glucose production. It is noteworthy that PKC- $\delta$  is also expressed in the duodenum (6,7) and that activation of duodenal mucosal PKC- $\delta$  inhibits glucose production (6). Although duodenal PKC- $\delta$  activation (6) or CCK action (2) is necessary for lipid sensing and sufficient to trigger a gut–brain–liver axis to lower glucose production, the interfunctional relationship between PKC- $\delta$  and CCK has yet to be established.

Two sets of studies report a potential mechanistic link between PKC and CCK. First, oleate activates PKC and stimulates CCK release in a CCK-secreting cell line (8,9). Conversely, CCK binds to the G-protein–coupled CCK-A receptor and stimulates the phospholipase C signaling pathway to activate PKC- $\delta$  and PKC- $\epsilon$  in pancreatic acinar cells (10–12). Together, these findings suggest that activation of duodenal PKC- $\delta$  may lie upstream (and/or downstream) of CCK signaling to trigger a neuronal network to regulate glucose production (Fig. 1A and Supplementary Fig. 1).

In this study, we attempt to clarify whether duodenal PKC- $\delta$  and CCK signaling converges (or diverges) to regulate glucose production. We first examined whether PKC- $\delta$  and CCK expression is colocalized in the duodenal mucosa. We then performed pharmacological and molecular gain- and loss-of-function experiments targeting PKC- $\delta$  and CCK-A receptor signaling within the intestine to determine whether duodenal PKC- $\delta$  activation lies upstream and/or downstream of CCK signaling to regulate glucose production.

## RESEARCH DESIGN AND METHODS

**Animal preparation.** The protocols were approved by the Institutional Animal Care Committee of the University Health Network. Male SD rats (250–300 g) were housed in individual cages and maintained on a standard light-dark cycle. The rats had access to chow and water *ad libitum* and were given 7 days to acclimatize before the designated surgeries were performed.

**Duodenal and intravenous cannulations.** Duodenal cannulation was performed where a catheter was inserted into the proximal duodenum (~1.5–2 cm downstream of the pyloric sphincter) (1,2). Intravenous catheters were placed into the internal jugular vein and carotid artery for infusion and blood sampling, respectively (1,2). Rats were monitored for daily food intake and body weight for 3 to 4 days after surgery and only underwent clamp studies when food intake and body weight were recovered back to at least 90% of basal values. A subgroup of rats with duodenal and intravenous catheters implanted were placed on a lard-oil enriched high-fat diet for 3 days (Purina Mills [TestDiet]; No. 0009389) that induces duodenal lipid/CCK resistance (1,2).

**Intraduodenal infusion and the pancreatic (basal insulin)-euglycemic clamp.** The clamp was performed as described (1,2) (Fig. 1B). The night before, rats were restricted to ~58 kcal of food to ensure the same nutritional post-absorptive status. At  $t = 0$  min, a primed continuous infusion of [ $^3$ H]glucose (bolus 40  $\mu$ Ci; 0.4  $\mu$ Ci/min) was given, and this rate was maintained throughout to assess changes in steady-state glucose kinetics based on tracer-dilution

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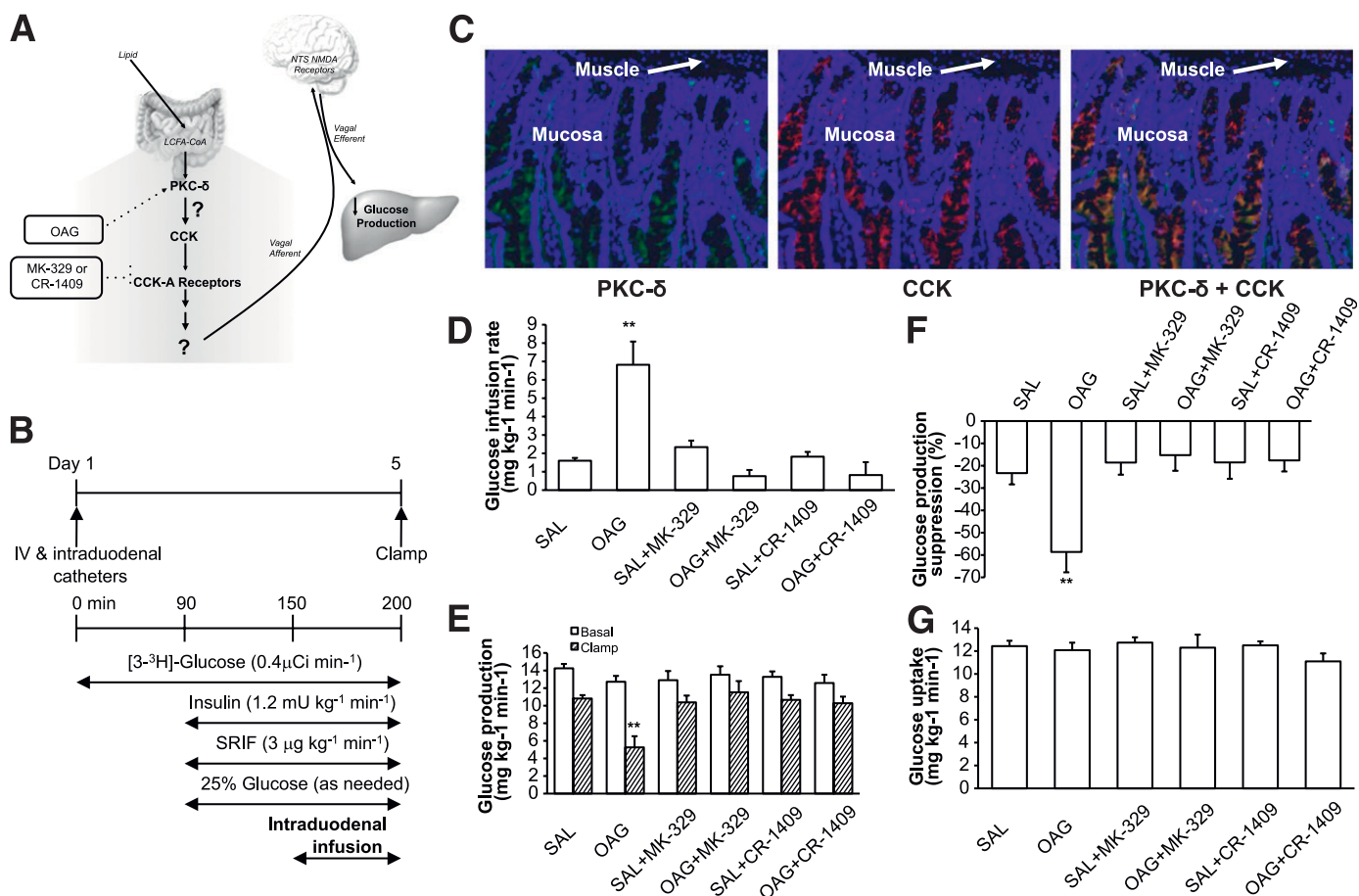
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**FIG. 1. A:** Schematic representation of the working hypothesis. OAG with or without MK-329 or CR-1409 infused through a duodenal catheter. **B:** Experimental procedure and clamp protocol. Duodenal catheter or venous and arterial catheters were implanted on day 1. On the evening before the pancreatic (basal insulin) clamp studies, all rats received a fixed portion of calories (~58 kcal) to ensure a comparable post-absorptive state at the start of the clamp experiments. **C:** Immunohistochemical stain of PKC- $\delta$  (left), CCK (center), and colocalization of PKC- $\delta$  and CCK (right) in the rat duodenum. PKC- $\delta$  stain is green, CCK stain is red, PKC- $\delta$  + CCK stain is orange, and nuclear stain is blue. Intraduodenal OAG infusion increased the glucose infusion rate (**D**) and decreased glucose production (**E** and **F**). Coinfusion of OAG with MK-329 or CR-1409 abolished the effect of OAG on the glucose infusion rate (**D**) and glucose production (**E** and **F**). **G:** Glucose uptake was comparable in all groups. \*\* $P < 0.01$  vs. all other groups. Saline ( $n = 6$ ), OAG ( $n = 7$ ), saline + MK-329 ( $n = 5$ ), OAG + MK-329 ( $n = 5$ ), saline + CR-1409 ( $n = 5$ ), and OAG + CR-1409 ( $n = 5$ ) are shown. Values are shown as mean + SEM. IV, intravenous; NMDA, N-methyl-D-aspartate; NTS, nucleus of the solitary tract; SAL, saline; SRIF, somatostatin. (A high-quality digital representation of this figure is available in the online issue.)

methodology. The clamp was started at  $t = 90$  min whereby a continuous infusion of insulin at 1.2 mU/kg/min and somatostatin at 3  $\mu$ g/kg/min were initiated. Somatostatin suppressed the plasma C-peptide level by >80% (basal:  $0.63 \pm 0.05$  nmol/L vs. clamp:  $0.12 \pm 0.03$ ;  $n = 14$ /group). A 25% glucose solution was infused at a variable rate to maintain euglycemia. The glucose infusion was adjusted every 10 min from  $t = 120$ –200 min. Intraduodenal infusions were initiated at  $t = 150$  min and maintained until  $t = 200$  min at 0.01 mL/min. Blood samples were taken at  $t = 90, 180,$  and 200 min to determine plasma insulin and C-peptide levels. Of note, during the clamps, plasma insulin levels (saline:  $0.7 \pm 0.2$ ; 1-oleoyl-2-acetyl-sn-glycerol [OAG]:  $0.7 \pm 0.1$ ; saline  $\pm$  MK-329:  $0.7 \pm 0.1$ ; OAG  $\pm$  MK-329:  $0.6 \pm 0.1$ ) were comparable among individual groups.

The following reagents were administered intraduodenally: 1) saline; 2) OAG–PKC activator (Calbiochem; 250  $\mu$ mol/L); 3) CCK-8 (sulfated)–CCK receptor agonist (Sigma-Aldrich; 35 pmol/kg/min); 4) MK-329–CCK-A receptor antagonist (Tocris Bioscience; 3.2  $\mu$ g/kg/min); 5) CR-1409–CCK-A receptor antagonist (Sigma-Aldrich; 29  $\mu$ g/kg/min), and 6) rottlerin–PKC- $\delta$  specific inhibitor (Calbiochem; 60  $\mu$ mol/L).

**Virus injection and surgery.** Adenovirus (Ad) application into the duodenum was performed as described (6). Ad expressing LacZ or the dominant negative form (DN) of PKC- $\delta$  (pfu/mL =  $4 \times 10^8$ ; 0.2 mL; gift from Dr. J. Soh, Biomedical Research Centre for Signal Transduction Network, Incheon, Korea) was injected into the duodenal lumen. A duodenal catheter was subsequently inserted through the injection site, followed by vascular cannulation using the same procedures described earlier. Animals were allowed to recover for 3 days before the clamp procedures. We have validated previously that duodenal injection of

Ad expressing LacZ selectively infected intestinal cells, but not hepatocytes, to a detectable level (3).

**Immunofluorescence.** Duodenal tissues were collected for immunofluorescence staining after animals were killed. After sectioning, the sections were postfixed with 4% PFA and Tween-20 (0.05%) and were blocked with 10% goat serum in phosphate-buffered saline for 1 h. To check for PKC- $\delta$  and CCK immunoreactivity, the sections were incubated with mouse anti-PKC- $\delta$  antibody (1:100) and rabbit anti-CCK-8 antibody (1:200) overnight at 4°C, respectively. The sections were incubated with goat anti-mouse antibody (1:500) and anti-rabbit antibody (1:200) for 1 h at room temperature. Immunolabeling was detected with the aid of a fluorescence microscope.

**Biochemical analysis.** Plasma glucose concentrations were measured using the glucose oxidase method (Glucose analyzer GM9; Analox Instruments). Plasma insulin and C-peptide concentrations were measured using RIA (Linc Research).

**Calculations.** Statistical analysis was performed using ANOVA followed by Tukey  $t$  test post hoc analysis. Significance was accepted at  $P < 0.05$ . Data are presented as means + SEM. The time period 60–90 min was averaged for the basal condition, and the time period 180–200 min was averaged for the clamp condition.

## RESULTS

Oleate activates PKC and stimulates CCK release within the same CCK-secreting cells (8,9). If duodenal CCK

signaling is necessary for PKC- $\delta$  activation to regulate glucose, PKC- $\delta$  should be colocalized with CCK in the duodenal mucosa. Immunostaining of CCK indicated that CCK was expressed along the duodenal mucosa (Fig. 1C) where the I cells that release CCK (13) are found. PKC- $\delta$  was expressed around the same area that contained CCK (Fig. 1C), and PKC- $\delta$  appeared to colocalize with CCK (Fig. 1C), suggesting a mechanistic link between PKC- $\delta$  and CCK.

To evaluate whether duodenal CCK signaling is required for duodenal mucosal PKC- $\delta$  activation to regulate glucose kinetics, we activated duodenal mucosal PKC- $\delta$  and inhibited the duodenal CCK-A receptor via intraduodenal coinfusion of OAG with either MK-329 or CR-1409 in normal rats during the clamps (Fig. 1A and B). When intraduodenal OAG was infused at a dose that activated duodenal mucosal PKC- $\delta$  activity (6), the glucose infusion rate required to maintain euglycemia was increased (Fig. 1D) as a result of an inhibition of glucose production (Fig. 1E and F). Coinfusion of either intraduodenal MK-329 or CR-1409 with OAG fully negated the ability of OAG to increase glucose infusion rate (Fig. 1D) and lower glucose production (Fig. 1E and F), whereas duodenal MK-329 or CR-1409 alone had no effects (Fig. 1D–F). Glucose uptake was comparable in all groups (Fig. 1G). These data indicate that activation of the duodenal CCK-A receptor is required for PKC- $\delta$  activation to lower glucose production, suggesting that PKC- $\delta$  activation lies upstream of CCK signaling.

It is equally possible that duodenal PKC- $\delta$  signaling lies downstream of CCK (Supplementary Fig. 1). We first tested the metabolic effects of duodenal CCK signaling (Supplementary Fig. 1), and consistent with a previous report (2), intraduodenal CCK-8 infusion increased the glucose infusion rate (Fig. 2A) and lowered glucose production (Fig. 2B and C) but did not alter glucose uptake (Fig. 2D). The intraduodenal PKC- $\delta$  inhibitor rottlerin infused at a dose that blocked the metabolic effects of OAG (6) failed to negate the ability of intraduodenal CCK-8 to regulate glucose kinetics (Fig. 2A–D).

To alternatively exclude the downstream signaling possibility of duodenal PKC- $\delta$  to CCK, duodenal mucosal PKC- $\delta$  activity was knocked down with a molecular approach (6) (Supplementary Fig. 1). We injected the Ad expressing LacZ (control) or the DN of PKC- $\delta$  directly into the lumen of the duodenum. Duodenal mucosal PKC- $\delta$  activity was knocked down by ~60% with intestinal Ad DN PKC- $\delta$ , and such inhibition of PKC- $\delta$  negated the ability of duodenal OAG to regulate glucose production (6). Intraduodenal CCK-8 was equally potent to increase the glucose infusion rate (Fig. 2E) and lower glucose production (Fig. 2F and G) in duodenal Ad LacZ-injected rats as compared with normal rats (Fig. 2A–C). The metabolic effects induced by intraduodenal CCK-8 preserved in rats injected with duodenal Ad DN PKC- $\delta$  (Fig. 2E–G). Glucose uptake was comparable in all groups (Fig. 2H). These data together indicate that duodenal PKC- $\delta$  signaling is not required for (or not downstream of) CCK to regulate glucose production.

Given that selective activation of duodenal mucosal PKC- $\delta$  enhances CCK signaling to regulate glucose production in normal rats, we tested whether activation of duodenal PKC- $\delta$  is sufficient to enhance CCK signaling to overcome CCK resistance in response to high-fat feeding (2). Intraduodenal OAG increased the glucose infusion rate (Fig. 3A) and lowered glucose production (Fig. 3B and C) independent of changes in glucose uptake (Fig. 3D) in normal rats. In contrast, intraduodenal OAG failed to alter

glucose kinetics (Fig. 3A–C) in rats fed with a high-fat diet. Thus, direct activation of duodenal PKC- $\delta$  fails to overcome CCK resistance in high fat-fed rats.

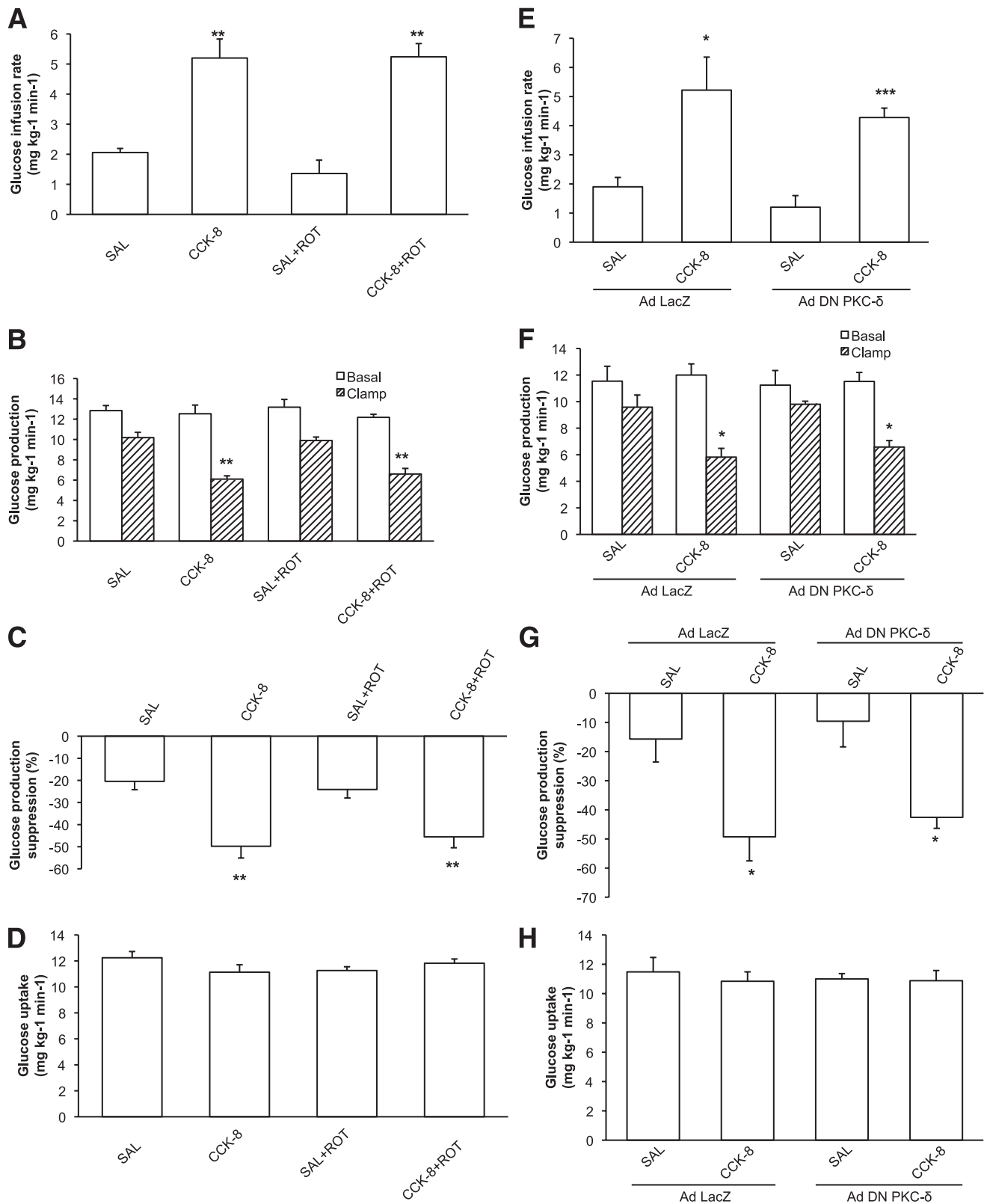
## DISCUSSION

Lipid metabolism within the duodenum triggers a neuronal network to lower glucose production in normal but not in high fat-fed rats (1). Given that diabetes and obesity are characterized by an excessive rate of glucose production (14,15), studies aimed to dissect the underlying mechanisms of duodenal lipid sensing could reveal novel therapeutic molecules that lower glucose production in diabetes and obesity (16).

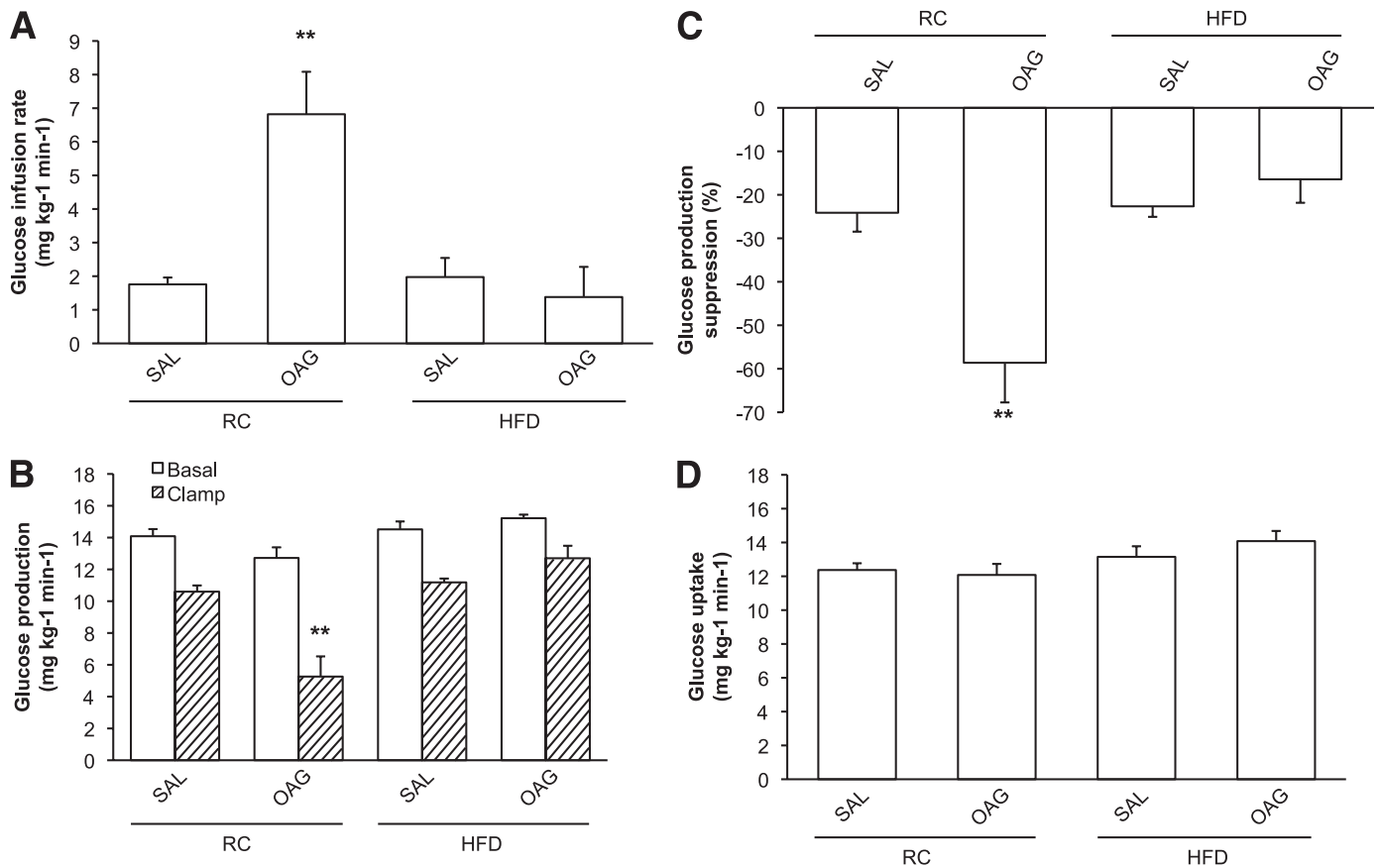
We here demonstrate that activation of the duodenal CCK-A receptor is necessary to mediate the metabolic effects of duodenal mucosal PKC- $\delta$  activation, thereby revealing a duodenal PKC- $\delta$  and CCK signaling axis that lowers glucose production in the presence of circulating basal insulin levels. Because activation of duodenal mucosal PKC- $\delta$  (6) or CCK-A receptors (2) is sufficient and necessary for lipid sensing to trigger a gut–brain–liver axis to lower glucose production in normal rats through the hepatic vagus, our data collectively indicate that metabolism of lipids within the duodenum activates mucosal PKC- $\delta$  and stimulates the release of CCK from the mucosal I cells, leading to the activation of CCK-A receptors (i.e., lipids→PKC- $\delta$ →CCK→CCK-A receptors) and lowering of glucose production. The mechanistic link(s) of the ability of PKC- $\delta$  to stimulate CCK release remains unknown. However, the potential involvement of the soluble NSF attachment protein receptor proteins (i.e., Munc18–1 and vesicle-associated membrane protein-2) warrants future investigation since PKC- $\delta$  enhances insulin secretion in association with increased phosphorylation of Munc18–1 in pancreatic  $\beta$ -cells (17) and vesicle-associated membrane protein-2 mediates CCK secretion in secreting tumor cell-1 cell lines (18).

Although the physiological relevance of the ability of the duodenal PKC- $\delta$ →CCK signaling axis to regulate glucose is supported by the fact that direct inhibition of either duodenal PKC- $\delta$  (6) or CCK-A receptors (2) disrupts glucose homeostasis during refeeding, the nutritional value of this newly described signaling axis remains to be explored. Experiments aimed to assess whether different types of long-chain fatty acid such as oleate or palmitate (via intraduodenal infusion or oral ingestion) trigger the PKC- $\delta$ →CCK signaling axis and regulate glucose production would begin to address the nutritional, physiological, and beneficial impact of this signaling pathway.

Rats acquired duodenal CCK resistance in response to high-fat feeding (2). Although the site(s) of the defect is unclear, evidence suggests that it lies within the signaling cascade of the duodenal CCK-A receptor since direct administration of the receptor agonist CCK-8 into the duodenum fails to lower glucose production in high fat-fed rats (2). Consistent with the fact that duodenal PKC- $\delta$  activation is upstream of CCK signaling and that diet-induced CCK resistance is postulated to lie within the downstream signaling cascade of CCK-A receptors, direct activation of duodenal mucosal PKC- $\delta$  still fails to overcome intestinal CCK resistance to lower glucose production. These findings strengthen the argument that duodenal lipid resistance is not a result of the inability of lipid to trigger signaling events (i.e., PKC- $\delta$  activation) to stimulate the local release of CCK from the mucosa, but rather, the downstream signaling cascade of CCK/CCK-A receptor.



**FIG. 2.** *A*: Intraduodenal CCK-8 infusion increased the glucose infusion rate, and the increase in glucose infusion rate remained when CCK-8 was coadministered with rotterlin (ROT). *B* and *C*: Glucose production during the clamps. *D*: Glucose uptake during the clamps. Saline (SAL;  $n = 7$ ), CCK-8 ( $n = 6$ ), rotterlin alone ( $n = 5$ ), and CCK-8 + rotterlin ( $n = 5$ ) are shown. \*\* $P < 0.01$  vs. saline. *E*: Intraduodenal infusion of CCK-8 increased the glucose infusion rate in both Ad LacZ- and Ad DN PKC- $\delta$ -injected rats. *F* and *G*: Glucose production during the clamps. *H*: Glucose uptake during the clamps. Saline + Ad LacZ ( $n = 5$ ), CCK-8 + Ad LacZ ( $n = 5$ ), saline + Ad DN PKC- $\delta$  ( $n = 5$ ), and CCK-8 + Ad DN PKC- $\delta$  ( $n = 5$ ) are shown. \*\*\* $P < 0.001$ , \* $P < 0.05$  vs. saline LacZ. Values are shown as mean + SEM.



**FIG. 3.** During the pancreatic clamps, rats that were placed on a high-fat diet (HFD) for 3 days failed to respond to intraduodenal OAG infusion to increase the glucose infusion rate (A) and lower glucose production (B and C) compared with rats fed regular chow. D: Glucose uptake was comparable in all groups. \*\* $P < 0.01$  vs. all other groups. Regular chow (RC) + saline (SAL;  $n = 7$ ), RC + OAG ( $n = 6$ ), HFD + saline ( $n = 6$ ), and HFD + OAG ( $n = 5$ ) are shown. Values are shown as mean + SEM.

Of note, although activation of duodenal PKC- $\delta$  is not required for CCK (i.e., CCK $\rightarrow$ CCK-A receptors $\rightarrow$ PKC- $\delta$ ) to regulate glucose production, our present data do not rule out the possibility that the CCK-A receptor may activate other isoforms of PKC via phospholipase C to regulate glucose production, which warrants future investigation. In summary, the current set of data unveils a novel duodenal PKC- $\delta$  and CCK signaling axis that regulates glucose production in vivo.

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No potential conflicts of interest relevant to this article were reported.

D.M.B. conducted and designed the experiments, performed data analyses, and wrote the manuscript. J.T.Y.Y.,

B.A.R., A.K., and G.W.C.C. assisted with experiments. T.K.T.L. supervised the project, designed the experiments, and edited the manuscript.

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