Hypothalamic Protein Kinase C Regulates Glucose Production

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OBJECTIVE—A selective rise in hypothalamic lipid metabolism and the subsequent activation of SUR1/Kir6.2 ATP-sensitive K^+ (K_{ATP}) channels inhibit hepatic glucose production. The mechanisms that link the ability of hypothalamic lipid metabolism to the activation of K_{ATP} channels remain unknown.

RESEARCH DESIGN AND METHODS—To examine whether hypothalamic protein kinase C (PKC) mediates the ability of central nervous system lipids to activate K_{ATP} channels and regulate glucose production in normal rodents, we first activated hypothalamic PKC in the absence or presence of K_{ATP} channel inhibition. We then inhibited hypothalamic PKC in the presence of lipids. Tracer-dilution methodology in combination with the pancreatic clamp technique was used to assess the effect of hypothalamic administrations on glucose metabolism in vivo.

RESULTS—We first reported that direct activation of hypothalamic PKC via direct hypothalamic delivery of PKC activator 1-oleoyl-2-acetyl-sn-glycerol (OAG) suppressed glucose production. Coadministration of hypothalamic PKC-δ inhibitor rottlerin with OAG prevented the ability of OAG to activate PKC-δ and lower glucose production. Furthermore, hypothalamic dominant-negative Kir6.2 expression or the delivery of the K_{ATP} channel blocker glibenclamide abolished the glucose production-lowering effects of OAG. Finally, inhibition of hypothalamic PKC eliminated the ability of lipids to lower glucose production.

CONCLUSIONS—These studies indicate that hypothalamic PKC activation is sufficient and necessary for lowering glucose production. ***Diabetes*** **57:2061–2065, 2008**

The hypothalamus senses nutrients and hormones to regulate energy and glucose homeostasis (1–9), but the associated central nervous system (CNS) sensing mechanisms remain unclear. A selective increase in long-chain fatty acyl-coenzyme A (LCFA-CoA) level in the hypothalamus leads to the activation of SUR1/Kir6.2-containing ATP-sensitive K^+ (K_{ATP}) channels and lowers glucose production (10). In contrast, an elevation of LCFA-CoA level in the liver actually increases glucose production during hyperinsulinemia (1). These observations led us to hypothesize that lipid-sensing mechanisms share similar biochemical (i.e., LCFA-CoA accumulation) but have opposing physiological mechanisms (i.e., glucose production regulation) in operation (1).

In the peripheral tissues such as the liver and muscle, an elevation of lipids (especially the long-chain fatty acids [LCFAs]) activates the novel isoforms of protein kinase C (PKC) (i.e., -δ, -ε, and -θ) to induce insulin resistance during hyperinsulinemic-euglycemic clamps (11–16). Although novel isoforms of PKC (especially -δ and -ε) are expressed in the brain (17), it is currently unknown whether LCFAs activate hypothalamic, novel isoforms of PKC to regulate glucose production. It has been reported that activation of PKC leads to phosphorylation of the conserved threonine residue (T180) in the pore-forming subunit Kir6.2 of the K_{ATP} channels in the pancreatic β-cells (18). These channels are expressed in both β-cells and neurons (18,19), and direct activation of the hypothalamic K_{ATP} channels has been shown to lower glucose production (19). Both the PKC-induced K_{ATP} channel activation (18) and hypothalamic K_{ATP} channels' regulation of glucose production (19) are blocked by pretreatment with the K_{ATP} channel blocker glibenclamide (18,19). It is possible that the mechanism of activation of K_{ATP} channels in the β-cells by PKC is also found in the hypothalamus.

Based on these independent yet parallel findings, we tested the hypothesis that activation of hypothalamic PKC is sufficient and necessary for CNS lipid-sensing mechanisms to lower glucose production and regulate glucose homeostasis (Fig. 1*A*).

RESEARCH DESIGN AND METHODS

We studied 8-week-old male Sprague-Dawley rats (Charles River Breeding Laboratories). Indwelling bilateral catheters (Plastics One, Roanoke, VA) were placed into the mediobasal hypothalamus (MBH) (3.1 mm posterior of bregma, 0.4 mm lateral from midline, and 9.6 mm below skull surface) 2 weeks before the experiments in vivo (20). One week later, catheters were placed in the internal jugular vein and the carotid artery for infusion and sampling during the clamp procedures (2). Recovery from surgery was monitored by measuring daily food intake and body weight gain in the 3–4 days preceding the infusion procedure. The study protocols were approved by the institutional animal care and use committee of the University Health Network in Toronto and the Albert Einstein College of Medicine in New York.

Clamp procedure. All the rats were restricted to 20 g of food the night before the experiments to ensure the same nutritional status. Infusion studies lasted a total of 360 min. At 0 min, MBH infusion of the various study solutions was initiated and maintained at a rate of 0.33 μl/h for 6 h. Study solutions consisted of 250 μmol/l PKC activator 1-oleoyl-2-acetyl-sn-glycerol (OAG) (dissolved in 5% DMSO), 250 μmol/l OAG plus 60 μmol/l bisindolylmaleimide (BIM) (general PKC inhibitor), 250 μmol/l OAG plus 60 μmol/l rottlerin (Rot) (specific PKC-δ inhibitor), 250 μmol/l OAG plus 100 μmol/l K_{ATP} channel blocker glibenclamide (dissolved in 5% DMSO), vehicle (either saline or 5%

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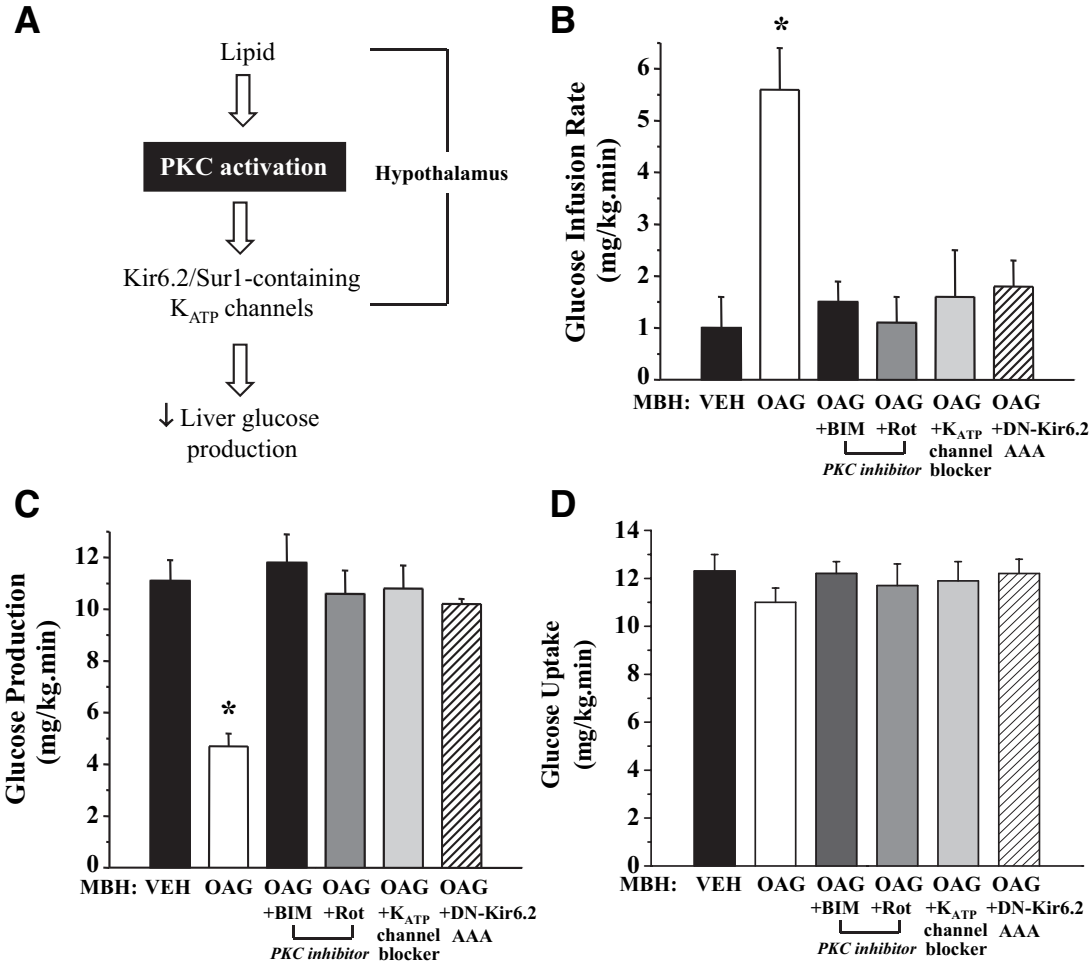

FIG. 1. Hypothalamic PKC activation lowers glucose production. *A*: Working hypothesis: lipids activate hypothalamic PKC to phosphorylate and activate the hypothalamic Kir6.2/SUR1-containing K_{ATP} channels to lower glucose production. Direct MBH administration of PKC activator OAG increased glucose infusion rate (*B*) and lowered glucose production (*C*) during the clamps. MBH OAG coinfused with general PKC inhibitor BIM ($n = 5$), specific PKC-δ inhibitor Rot ($n = 6$), or K_{ATP} channel blocker glibenclamide ($n = 5$) or in MBH DN Kir6.2 AAA-injected rats ($n = 5$) failed to increase glucose infusion rate (*B*) and lower glucose production (*C*). *D:* Glucose uptake was comparable in all groups. MBH vehicle (VEH) ($n = 6$) consisted of MBH saline ($n = 3$) and MBH 5% DMSO ($n = 3$). MBH OAG ($n = 7$) consisted of MBH OAG in normal rats ($n = 4$) and in MBH GFP-injected rats ($n = 3$). *$P < 0.001$ (ANOVA) and $P < 0.01$ vs. other individual groups.

DMSO), 60 μmol/l BIM, 60 μmol/l Rot, or 100 μmol/l glibenclamide. After 2 h of MBH infusion, a primed continuous intravenous infusion of 3-^{3}H-glucose (40 μCi bolus, 0.4 μCi/min; Perkin Elmer) was begun and maintained throughout the study to assess glucose kinetics in vivo.

In a different group of rats, intravenous saline or 20% Intralipid (mixed with 20 units/ml of heparin, 0.4 ml/h; Baxter Healthcare Corporation) infusion was initiated at 120 min and maintained throughout the experiments. Intralipid infused at this rate elevates plasma free fatty acid and hypothalamic LCFA-CoA levels by ~1.5- to 2.0-fold (10). Samples for determination of ^{3}H glucose–specific activity were obtained at 10-min intervals. For the final 2 h of the infusion study, a pancreatic-euglycemic clamp was performed to evaluate the effect of MBH treatments on glucose metabolism independent of changes in glucoregulatory hormones. This involved continuous infusions of insulin (0.8 mU · kg^{-1} · min^{-1} for MBH OAG studies and 1.5 mU · kg^{-1} · min^{-1} for intravenous intralipid infusion studies) and somatostatin (3 μg · kg^{-1} · min^{-1}) to inhibit endogenous insulin and glucagon secretions and a variable infusion of a 25% glucose solution that was started and periodically adjusted to maintain the plasma glucose concentration similar to the basal levels (210–240 min).

The data for glucose infusion rate, glucose production, and glucose uptake were presented as an average of values obtained from the final 30 min (300–360 min) of the clamp studies. MBH Rot, BIM, and glibenclamide alone did not affect glucose kinetics (data not shown). In a separate set of experiments, we performed clamps infused with a higher dose of insulin (1.2 instead of 0.8 mU · kg^{-1} · min^{-1}) and administered vehicle ($n = 5$) or OAG ($n = 4$) into the MBH. We found that insulin clamped at 1.2 mU · kg^{-1} · min^{-1} normally suppressed glucose production from ~11 to mean ± SD 7.3 ± 0.4 mg · kg^{-1} · min^{-1} in MBH vehicle–treated rats. MBH OAG further suppressed glucose production to 2.9 ± 0.9 mg · kg^{-1} · min^{-1} ($P < 0.01$ vs. MBH vehicle) during the clamps infused with the higher dose of insulin. The effects of MBH OAG on glucose production suppression was additive to peripheral insulin's effect on the liver, although the magnitude of the drop (~4.5 mg · kg^{-1} · min^{-1}) was similar compared with that observed during the pancreatic basal insulin clamps (Fig. 1*C*). We also performed additional pancreatic basal insulin clamps with intracerebroventricular (icv) OAG ($n = 4$) versus vehicle ($n = 5$). The data indicated that icv OAG suppressed glucose production (5.9 ± 0.4 [icv OAG, $n = 4$] vs. 10.8 ± 0.8 mg · kg^{-1} · min^{-1} [icv vehicle, $n = 5$], $P < 0.01$) to a similar extent as it was suppressed MBH OAG (Fig. 1*C*). At the end of all studies, rats were anesthetized (ketamine 150 mg/kg) and tissue samples were freeze-clamped with precooled aluminum tongs in situ. All tissue samples were stored at −80°C for further analysis. Harvard Apparatus PHD 2000 infusion pumps were used for all infusions during the clamps.

Mediobasal hypothalamic wedge sampling. The MBH was sampled by dissecting a wedge of tissue including the entire mediolateral and dorsoventral extent of the arcuate nuclei while minimizing ventromedial nucleus tissue.

Adenovirus injection. Immediately poststereotaxic surgery, MBH cannulated rats were administered 3 μl of adenovirus containing dominant-negative (DN) Kir6.2 AAA-AV (21) (3.1×10^{10} plaque-forming units/ml) or green fluorescent protein (GFP) (3.0×10^{10} plaque-forming units/ml) per side of cannula, as previously described (22). Five days later, the rats underwent vascular surgeries and clamp studies, with MBH OAG administrations performed 4 days later. Preliminary studies ($n = 3$) indicate that MBH OAG similarly lowered glucose production in GFP-injected rats compared with noninjected rats ($n = 4$). Thus, the studies were combined and presented as a single MBH OAG group in Fig. 1*B* and *C*.

Hypothalamic PKC isoform translocation. The plasma translocation of the novel isoforms of PKC was assessed by comparing immunoblots of the cytosolic- and membrane-associated fractions obtained 5 min after the MBH DMSO, OAG, OAG + Rot, and Rot treatments in vivo. It is important to point out that Rot has previously been shown to inhibit PKC-δ translocation in neuronal cells (23). MBH wedges (~8 mg) were homogenized using a handheld glass homogenizer in lysis buffer A (20 mmol/l 3-(N-morpholino) propanesulfonic acid [MOPS], 2 mmol/l EDTA, 0.32 mmol/l sucrose, 30 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 2 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonylfluoride, 3 mmol/l benzamidine, 5 μmol/l pepstatin A, and 10 μmol/l leupeptin). The samples were centrifuged at 500*g* to pellet out the nuclear fraction, and the supernatant was then cetrifuged at 100,000*g* for 60 min at 4°C. The supernatant was retained as the cytosolic fraction. The pellet fraction was washed in lysis buffer A and then resuspended in lysis buffer B (lysis buffer A plus 1% Triton X-100). The samples were incubated for 60 min on ice and then centrifuged at 100,000*g* for 60 min at 4°C. The supernatant provided the solubilized membrane fraction.

The protein concentration of all samples was determined by detergent-compatible microbicinchoninic acid assay (microBCA, Pierce) using serum albumin as the standard. Eight micrograms of protein in all samples were mixed with LDS sample loading buffer and sample reducing agent (Invitrogen) and heated to 70°C for 10 min. The mixture was then subjected to electrophoretic separation (4–12% bis-tris) and transferred to polyvinylidine fluoride membranes. The membranes were incubated for 1 h in Li-cor blocking buffer at room temperature and then incubated overnight at a concentration of 1:1,000 with affinity-purified polyclonal antibodies specific for the various novel isoforms of PKC (Santa Cruz). Consistent with previous findings (17), no bands for PKC-θ in the MBH wedges were detected using high or low dilution of the antibody. Membranes were washed with Tris-buffered saline plus Tween and then incubated for 1 h at room temperature with immunoreactive-dye–conjugated IgG (Rockland). The membranes were washed again with Tris-buffered saline-Tween and then rinsed with PBS and scanned on the Odyssey Infrared imaging system (Li-Cor). The immunoreactive bands were quantified using the analytical programs on the Odyssey system.

Statistical analysis. Statistical analysis was done by ANOVA to compare across the groups followed by Tukey post hoc test to compare between groups. Statistical analysis was accepted as significant with a P value <0.05. Data are presented as means + SE.

RESULTS

To examine the glucoregulatory effects of hypothalamic PKC activation, we infused PKC activator OAG (250 μmol/l) into the MBH of conscious unrestrained rats in vivo. The effects on glucose production and glucose uptake induced by MBH OAG administration were assessed by the tracer-dilution methodology and the pancreatic basal insulin clamp technique as previously described (2). During the clamps, MBH OAG increased exogenous glucose infusion rate by ~2.5-fold, compared with MBH vehicle, which maintained euglycemia (Fig. 1*B*). The increase in glucose infusion rate was fully accounted for by an inhibition of glucose production (Fig. 1*C*) because the rate of glucose uptake remained unchanged (Fig. 1*D*), indicating that direct hypothalamic PKC activation lowered glucose production.

We then coinfused MBH OAG with the general PKC inhibitor BIM (60 μmol/l) or with the specific PKC-δ inhibitor Rot (60 μmol/l). We found that inhibition of hypothalamic PKC, or more specifically PKC-δ, with both inhibitors diminished the ability of MBH OAG to increase exogenous infusion rate and to lower glucose production (Fig. 1*B* and *C*). We administered OAG into the MBH in a different group of rats at the same concentration that was used in the clamp studies, and we found that MBH OAG selectively induced hypothalamic PKC-δ plasma membrane translocation (an accepted marker for PKC activation [24,25]) (Fig. 2*A*). Importantly, MBH OAG–induced PKC-δ membrane translocation was blocked by the coadministration of PKC-δ inhibitor Rot at the same concentration that blocked the ability of MBH OAG to lower glucose production (Fig. 1*C*). MBH OAG (with or without Rot) decreased PKC-ε content in the cytosolic fraction but with no parallel increase in the membrane fraction (Fig. 2*B*). Because we hypothesized that the activation of hypothalamic PKC leads to the activation/phosphorylation of the K_{ATP} channels that are located in the plasma membrane, we postulate that hypothalamic PKC-δ but not PKC-ε regulates glucose production. However, this remains to be proven. Together, these data suggest that hypothalamic PKC-δ activation lowered glucose production.

Hypothalamic K_{ATP} channels mediate CNS nutrient sensing to regulate glucose production (1,20). Given the ability of PKC to phosphorylate and activate the Kir6.2-SUR1 K_{ATP} channels (18) that are expressed in both pancreatic β-cells and neurons (19), we first tested whether activation of hypothalamic K_{ATP} channels is required for PKC to lower glucose production with a pharmacological approach. We coinfused MBH OAG with low doses of the K_{ATP} channel blocker glibenclamide (100 μmol/l), and this fully reversed the ability of MBH OAG to increase glucose infusion rate and lower glucose production (Fig. 1*B* and *C*). We next tested the role of hypothalamic K_{ATP} channels with a molecular approach. DN Kir6.2 channel expression induced by an adenovirus (adenovirus-expressing Kir6.2 AAA) disrupts the Kir6.2 channel current by more than 90% in cardiac myocytes (21). The DN Kir6.2 AAA adenovirus expresses an AAA mutant subunit of Kir6.2 that co-assembles with endogenous Kir6.2 and prevents the fully assembled K_{ATP} channel complex from conducting potassium current (21). We injected the DN Kir6.2 AAA adenovirus or GFP previously into the MBH of rats and performed the clamp procedure with MBH OAG administration. MBH OAG failed to increase glucose infusion rate and lower glucose production in the DN Kir6.2 AAA previously rats compared with the GFP adenovirus rats (Fig. 1*B* and *C*). These data collectively indicate that the hypothalamic Kir6.2/SUR1-containing K_{ATP} channel is required for PKC to lower glucose production. Future studies are needed to clarify whether hypothalamic PKC directly phosphorylates/activates the K_{ATP} channels to regulate glucose production.

During the pancreatic basal insulin clamps, an elevation of plasma LCFA increases gluconeogenesis but not glucose production because of an inhibition of glycogenolysis (10,26,27). The activation of hypothalamic K_{ATP} channels by an approximately twofold elevation of circulating LCFA (induced by intravenous intralipid infusion) was recently demonstrated to inhibit glycogenolysis and compensate for the induction in gluconeogenesis (10). In light of the fact that *1*) LCFAs activate PKC in liver and muscle (11–16) and *2*) hypothalamic PKC-K_{ATP} channel activation lowers glucose production (demonstrated in the current study), we tested, using the same intravenous intralipid-infused model (10), whether activation of hypothalamic PKC is required for circulating LCFA to lower glucose production. We inhibited hypothalamic PKC activation with either MBH BIM (60 μmol/l) or Rot (60 μmol/l) administration to rats that received intravenous intralipid. MBH BIM or Rot decreased the exogenous glucose infusion rate required to maintain euglycemia in rats that received lipid infusion (Fig. 2*C*). The decrease in glucose infusion rate was fully accounted for by an elevation of glucose production, since glucose uptake was unchanged (Fig. 2*D* and *E*). Together, these data indicate that hypothalamic PKC activation is required for circulating lipids to lower glucose production.

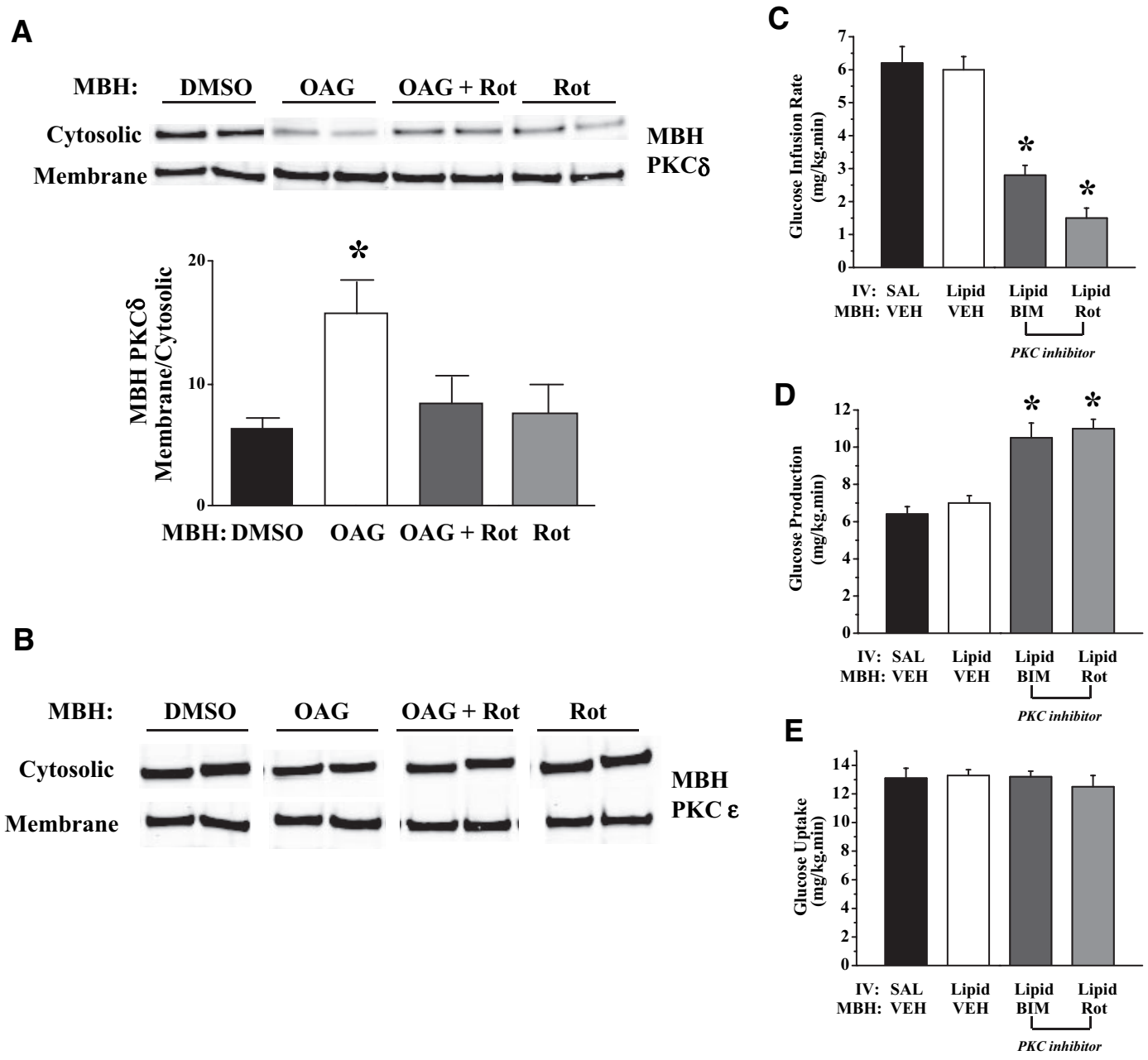

FIG. 2. Lipids activate hypothalamic PKC to lower glucose production. *A*: MBH OAG administration selectively induced hypothalamic PKC-δ plasma membrane translocation (n = 5 per group; *P < 0.05 vs. other individual groups; *P < 0.05 [ANOVA]), and this MBH OAG-induced hypothalamic PKC-δ translocation was reversed by coadministration of MBH PKC-δ inhibitor Rot. *B*: MBH OAG (with or without Rot) decreased the cytosolic content but did not increase the membrane content of hypothalamic PKC-ε. MBH BIM (n = 5) or Rot (n = 6) administration decreased the glucose infusion rate (*C*) and increased glucose production (*D*) in intravenous (IV) intralipid-infused rats. MBH VEH plus intravenous saline (n = 6); MBH vehicle (VEH) plus intravenous lipid (n = 6). *E*: Glucose uptake was comparable in all groups. *P < 0.001 (ANOVA) and P < 0.001 vs. the corresponding controls.

Finally, we tested whether activation of hypothalamic PKC lowers glucose production in an early-onset (3 days of high-fat feeding) diet-induced hepatic insulin resistance model (2,28,29). We found that during the pancreatic basal insulin clamps, MBH OAG suppressed glucose production in high-fat–fed rats ($5.7 + 0.7\ mg \cdot kg^{-1} \cdot min^{-1}$ [MBH OAG; $n = 5$] vs. $11.5 + 0.5\ mg \cdot kg^{-1} \cdot min^{-1}$ [MBH vehicle; $n = 5$], $P < 0.001$) to a similar extent as that observed in rats given a regular diet (Fig. 1*C*). These data indicate that direct PKC activation bypasses lipid-sensing defect(s) in the hypothalamus (28) to lower glucose production in the early onset of diet-induced insulin resistance.

DISCUSSION

Our data first support the notion that activation of hypothalamic PKC is sufficient and necessary for CNS lipid-sensing mechanisms to lower glucose production through the activation/phosphorylation of hypothalamic Kir6.2/SUR1-containing K_{ATP} channels. However, much work is needed to further dissect the mechanisms in the hypothalamus or in other parts of this brain-liver axis that regulates glucose production. Second, these data support the working hypothesis that lipid-sensing mechanisms in the brain and liver share biochemical mechanisms but have opposing physiological mechanisms that regulate glucose pro-

duction (1). Finally, our findings reveal hypothalamic PKC as a potential therapeutic target for lowering glucose levels in diabetes and obesity.

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