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Effect of Guanylate Cyclase-C Activity on Energy and Glucose Homeostasis

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OBESITY STUDIES

Uroguanylin is a gastrointestinal hormone primarily involved in fluid and electrolyte handling. It has recently been reported that prouroguanylin, secreted postprandially, is converted to uroguanylin in the brain and activates the receptor guanylate cyclase-C (GC-C) to reduce food intake and prevent obesity. We tested central nervous system administration of two GC-C agonists and found no significant reduction of food intake. We also carefully phenotyped mice lacking the GC-C receptor and found them to have normal body weight, adiposity, and glucose tolerance. Interestingly, uroguanylin knockout mice had a small but significant increase in body weight and adiposity that was accompanied by glucose intolerance. Our data indicate that the modest effects of uroguanylin on energy and glucose homeostasis are not mediated by central GC-C receptors.

Prouroguanylin is a peptide secreted primarily from the small intestine and is cleaved to the active hormone uroguanylin at the site of action (1). Uroguanylin and its transmembrane receptor guanylate cyclase-C (GC-C or *GUCY2C*) are predominantly found in intestinal epithelial cells and function to regulate intracellular cyclic guanosine monophosphate (cGMP) production (2). The GC-C receptor is also bound by the related intestinal hormone guanylin and bacterial heat-stable enterotoxins (STs) (3,4). Ligand-induced activation of GC-C regulates electrolyte and fluid secretion into the intestinal lumen (5). Further, this receptor regulates additional processes in the healthy and diseased gut, including epithelial cell proliferation (6), colonic inflammation and infection (7–9), and sodium homeostasis (10,11).

In addition to the secretion of prouroguanylin into the gut lumen, prouroguanylin is released into the circulation and targets multiple tissues, including the kidney and brain (10,12). Notably, uroguanylin regulates electrolyte secretion in the kidney nephron via both GC-C–dependent and GC-C–independent mechanisms (13,14). Valentino et al. (15) recently reported that intestinal prouroguanylin, released postprandially, may be a satiation factor that reduces feeding behavior in mice by acting at GC-C receptors in the hypothalamus.

The hypothesis that uroguanylin and GC-C are involved in the normal regulation of energy intake has tangential support from invertebrate studies. In both *Drosophila* (16) and *Caenorhabditis elegans* (17), cGMP signaling is an important factor influencing feeding behavior. In addition, other central cGMP activators, including the natriuretic peptides and nitric oxide, also alter energy balance by suppressing food intake (18,19).

Because of the potential importance of a cGMP-based central nervous system circuit regulating food intake and energy homeostasis, we sought to determine the effects of central nervous system administration as well as loss of function of both the receptor and uroguanylin on energy balance regulation. In a series of experiments, we observed no effect on food intake or body weight following administration of uroguanylin—or the GC-C agonist ST—into the brain. Further, there were no differences in body weight, adiposity, or glucose tolerance between GC-C–deficient mice and their littermate controls. However, uroguanylin-deficient mice had increased weight gain, adiposity, and glucose intolerance. While our data do not support a role for GC-C and uroguanylin in the hypothalamic control of

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food intake, uroguanylin does appear to influence energy and glucose homeostasis potentially via mechanisms that are independent of the GC-C receptor.

RESEARCH DESIGN AND METHODS

Animals and Housing

Adult male Long-Evans rats (250–300 g; Harlan Laboratories, Harlan, IN) were housed in individual tub cages. GC-C ($^{-/-}$ and $^{+/+}$) and uroguanylin (UGN; $^{-/-}$ and $^{+/+}$) mice were bred from heterozygous pairs on a >10 generation C57Bl/6 background and maintained 2–3 per cage until the final 4 weeks of the experiment when they were separated to perform a mixed-meal tolerance test. All animals were maintained on a 12:12-h light:dark cycle in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facilities of the Metabolic Diseases Institute of the University of Cincinnati. Except where specified, animals had ad libitum access to water and pelleted diet. Rats were maintained on a low-fat chow diet (3.1 kcal/g; percentage of energy from fat, 17%; carbohydrate, 58%; protein, 25%; Teklad Rat Chow, Harlan Laboratories, Madison, WI). Mice were maintained on the low-fat chow diet until 10 weeks of age and were then provided with high-fat diet (HFD; n = 40; 4.54 kcal/g; percentage of energy from fat, 40%; carbohydrate, 45%; protein, 15%; OpenSource Diets, Research Diets Inc., New Brunswick, NJ). All protocols were approved by the University of Cincinnati Animal Care and Use Committee.

Third-Ventricular Cannulation

Surgery was performed using sterile techniques as previously described (20). Briefly, rats (n = 22) were anesthetized using ketamine (70 mg/kg) and xylazine (6 mg/kg) intraperitoneally, shaved, and positioned in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). A stainless-steel cannula (Plastics One, Chantilly, VA) was positioned 7.5 mm ventral to the dura on the midline 2.2 mm posterior to bregma, and the cannula was fixed to the skull with dental acrylic anchored by screws. Placement of the cannula was confirmed behaviorally by injecting 10 ng of angiotensin II (American Peptide, Sunnyvale, CA) in 1 μL normal saline through the cannula. Rats that consumed >5 mL of water within 30 min were considered to have a viable cannula; two rats were excluded.

Third-Ventricular Cannulation Injections and Food Intake

Animals were handled and weighed daily. Rats had chow removed for the final 4 h of the light phase on both the day prior to and the day of injections. Injections were performed in a repeated-measures counter-balanced manner with each injection made at least 4 days apart so that each animal received 2-μL injections of uroguanylin (5, 10, 25, or 50 μg; Sigma-Aldrich, St. Louis, MO) and a saline control. A dose response was performed due to no published data relating to central uroguanylin administration. Animals were then administered the GC-C activator ST (1 μg; Sigma-Aldrich) and exendin-4 (1 μg) as a positive control or a saline control, also in a repeated-measures counter-balanced manner. Injections were given 30 min before the onset of dark. Food hoppers were returned at the onset of dark, and intake was assessed 1, 2, 4, and 24 h after food return. Body weight was measured prior to injection and after 24 h.

Diet-Induced Obesity in GC-C and UGN Mice

At 10 weeks of age, male mice (n = 10–12/genotype) were provided with a HFD for 16 weeks. After 12 weeks on the HFD, mice were separated into individual cages to measure food intake. Mixed-meal tolerance was performed after 14 weeks on the HFD. Body composition analysis was performed using nuclear magnetic resonance (NMR) prior to and after 16 weeks on the HFD.

NMR Body Composition Analysis

Body composition (fat mass and lean mass) was assessed using NMR in conscious mice (EchoMRI, Houston, TX).

Mixed-Meal Tolerance Test

Following a 4-h fast, mice were gavaged with a mixed meal (Ensure, Abbott Nutrition, North Chicago, IL; 200 μL/mouse). Blood glucose was assessed at baseline, 15, 30, 45, 60, 120, and 240 min (Accu-Chek, Roche Diagnostics, Indianapolis, IN). Insulin was assessed at baseline and 15 min by ELISA (Crystal Chem, Downers Grove, IL).

cGMP Activity in Hypothalamic Tissue

cGMP activity was assessed by enzyme immunosorbent assay using hypothalamic tissue dissected 30 min after third-ventricular cannulation (I3VT) of either 10 μg uroguanylin, 1 μg ST, or vehicle. The hypothalamic block was dissected between the optic chiasm, and the dorsal edge of the fornix tissue was homogenized in 5% trichloroacetic acid, centrifuged, and extracted with water-saturated ether. Samples were acetylated and then assayed according to the manufacturer's directions (Cayman Chemical, Ann Arbor, MI).

Gene Expression

Gene expression was assessed using RT-PCR similar to previous reports in rat hypothalamic tissue. Briefly, RNA was extracted from tissue using trireagent, assessed for quality using a spectrophotometer, and converted to cDNA. Expression of GC-C and uroguanylin were determined using gene-specific probes in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Statistical Analyses

Data were analyzed using repeated-measures ANOVA, one-way ANOVA, or repeated-measures t tests as appropriate. Post hoc Tukey tests were performed where significant interactions were observed in ANOVA. Significance was accepted at $P < 0.05$, with data reported as mean ± SEM.

RESULTS

I3VT Uroguanylin and GC-C Agonism Does Not Inhibit Food Intake or Reduce Body Weight

I3VT uroguanylin, at increasing doses (0–50 μg), did not alter food intake in rats after 1, 2, 4, or 24 h following

infusion relative to vehicle, saline (Fig. 1*A*). Similarly, 24-h body weight was not affected by I3VT uroguanylin (Fig. 1*B*). To determine if I3VT uroguanylin has a more transient effect, food intake was assessed 15 and 30 min following I3VT uroguanylin (25 μg) in an additional experiment, but, again, no difference was observed between uroguanylin and saline (Fig. 1*C*). I3VT administration of the GC-C agonist ST (1 μg) also had no effect on food intake, whereas I3VT administration of exendin-4 (1 μg), a GLP-1 agonist, produced a marked reduction of food intake at 1, 2, 4, and 24 h in the same animals ($P < 0.05$) (Fig. 1*D*). Body weight was reduced only following exendin-4 infusion ($P < 0.05$) (Fig. 1*E*).

GC-C Receptor Is Present in the Hypothalamus and cGMP Production Is Increased in Response to I3VT Uroguanylin and ST

A failure to alter food intake could result from the administered compound not activating hypothalamic GC-C receptors. Initially, we used real-time RT-PCR to confirm the presence of GC-C receptor mRNA in hypothalamic tissue (no detectable uroguanylin mRNA was found) (Fig. 1*F*). I3VT administration of uroguanylin (10 μg) or ST (1 μg) increased cGMP production in hypothalamic tissue (Fig. 1*G*), demonstrating that the administered ligands were biologically active and capable of activating GC-C receptors.

Diet-Induced Obesity Develops Normally in GC-C$^{-/-}$ Mice But Is Modestly Increased in UGN$^{-/-}$ Mice

Relative to wild-type littermate controls, GC-C–deficient (GC-C$^{-/-}$) and uroguanylin-deficient (UGN$^{-/-}$) mice displayed no change of body weight (Fig. 2*A*), fat mass (Fig. 2*B*), or lean mass at 8 weeks of age when maintained on low-fat chow (Fig. 2*C*). When maintained on HFD, GC-C$^{-/-}$ mice had equivalent body weight gain as their littermates (GC-C$^{+/+}$) (Fig. 2*D*). UGN$^{-/-}$ mice had greater weight gain than UGN$^{+/+}$ mice ($P < 0.05$) (Fig. 2*E*), indicating an increased propensity to develop diet-induced obesity. Adipose tissue was increased comparably in GC-C$^{-/-}$ and GC-C$^{+/+}$ mice after 16 weeks on a HFD. In contrast, UGN$^{+/+}$ had significantly less adipose tissue compared with UGN$^{-/-}$ mice ($P < 0.05$) (Fig. 2*F*). Lean mass was not affected by GC-C or UGN genotype (Fig. 2*G*). Mean daily food intake was not affected by GC-C genotype (GC-C$^{+/+}$ 3.26 ± 0.32 g/day; GC-C$^{-/-}$ 3.23 ± 0.24 g/day) but was moderately increased in UGN $^{-/-}$ mice (3.37 ± 0.21 g/day) relative to their controls (2.80 ± 0.18 g/day; $P < 0.05$).

GC-C$^{-/-}$ Mice Have Normal Mixed-Meal Tolerance, Whereas UGN$^{-/-}$ Mice Have Significant Insulin Resistance

Fasting glucose was not different in GC-C$^{-/-}$ or UGN$^{-/-}$ mice relative to their littermate controls (Fig. 3*A*). Following an intragastric mixed-meal gavage, blood glucose excursions

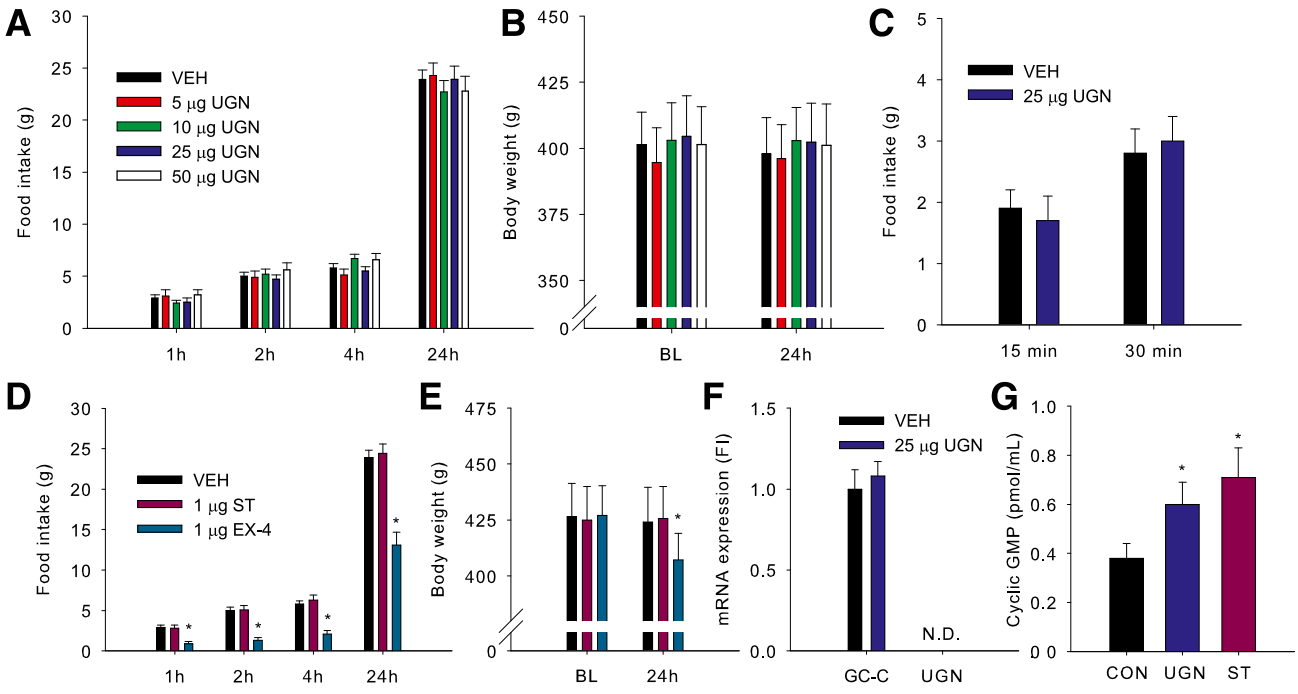

Figure 1—I3VT UGN and the bacterial enterotoxin ST do not inhibit food intake or reduce body weight. No reduction of food intake was observed 1, 2, 4, or 24 h after increasing doses of UGN (*A*). Body weight at 24 h was also unaffected relative to baseline (*B*). Additionally, there was no short-term impact of UGN on food intake at 15 or 30 min (*C*). The GC-C agonist, ST, also produced no change in food intake (*D*) or body weight (*E*) despite the positive control, exendin-4, reducing both. Transcripts for GC-C, but not UGN, were present in hypothalamic tissue (*F*). cGMP was produced in response to I3VT UGN or ST, indicating presence and activity of the receptor in the hypothalamus independent of energy balance (*G*). *$P < 0.05$ relative to VEH (*D*), BL (*E*), or CON (*G*). BL, baseline; CON, control; EX-4, exendin-4; FI, food intake; N.D., not detected; VEH, vehicle.

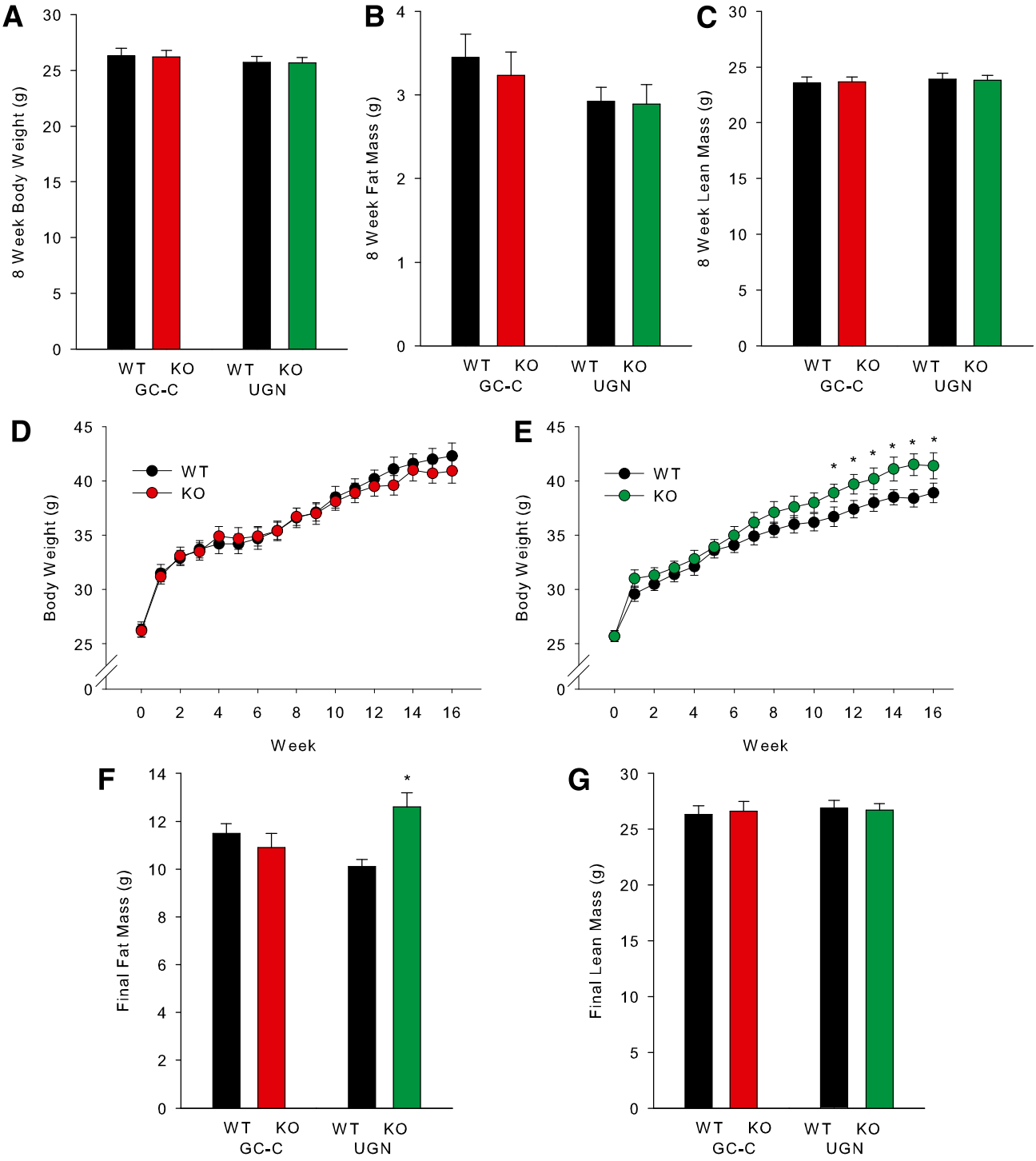

Figure 2—Diet-induced obesity develops normally in GC-C–deficient mice but is modestly increased in UGN-deficient mice. At 8 weeks, GC-C– and UGN-deficient mice maintained on a low-fat chow diet had no body weight (*A*), fat mass (*B*), or lean mass (*C*) differences relative to wild-type controls. When maintained on HFD, GC-C–deficient mice had similar weight gain to wild-type littermates over 16 weeks (*D*). UGN-deficient mice on HFD had increased weight gain relative to wild-type littermates from week 11 (*E*). The increased body weight of UGN-deficient mice was reflected in an increased fat mass after 16 weeks on HFD (*F*), whereas lean mass was unaffected by genotype (*G*). *$P < 0.05$ relative to WT control. KO, knockout; WT, wild type.

were comparable between GC-C$^{-/-}$ and GC-C$^{+/+}$ mice (Fig. 3*B*). UGN$^{-/-}$ mice had a significantly greater glucose excursion than UGN$^{+/+}$ mice ($P < 0.05$) (Fig. 3*C*). Fasting insulin levels were unaffected by GC-C or UGN genotype (Fig. 3*D*).

DISCUSSION

In contrast to a previous report in the mouse (15), acute I3VT administration of uroguanylin or the GC-C agonist and enterotoxin, ST, elicited no changes of food intake or

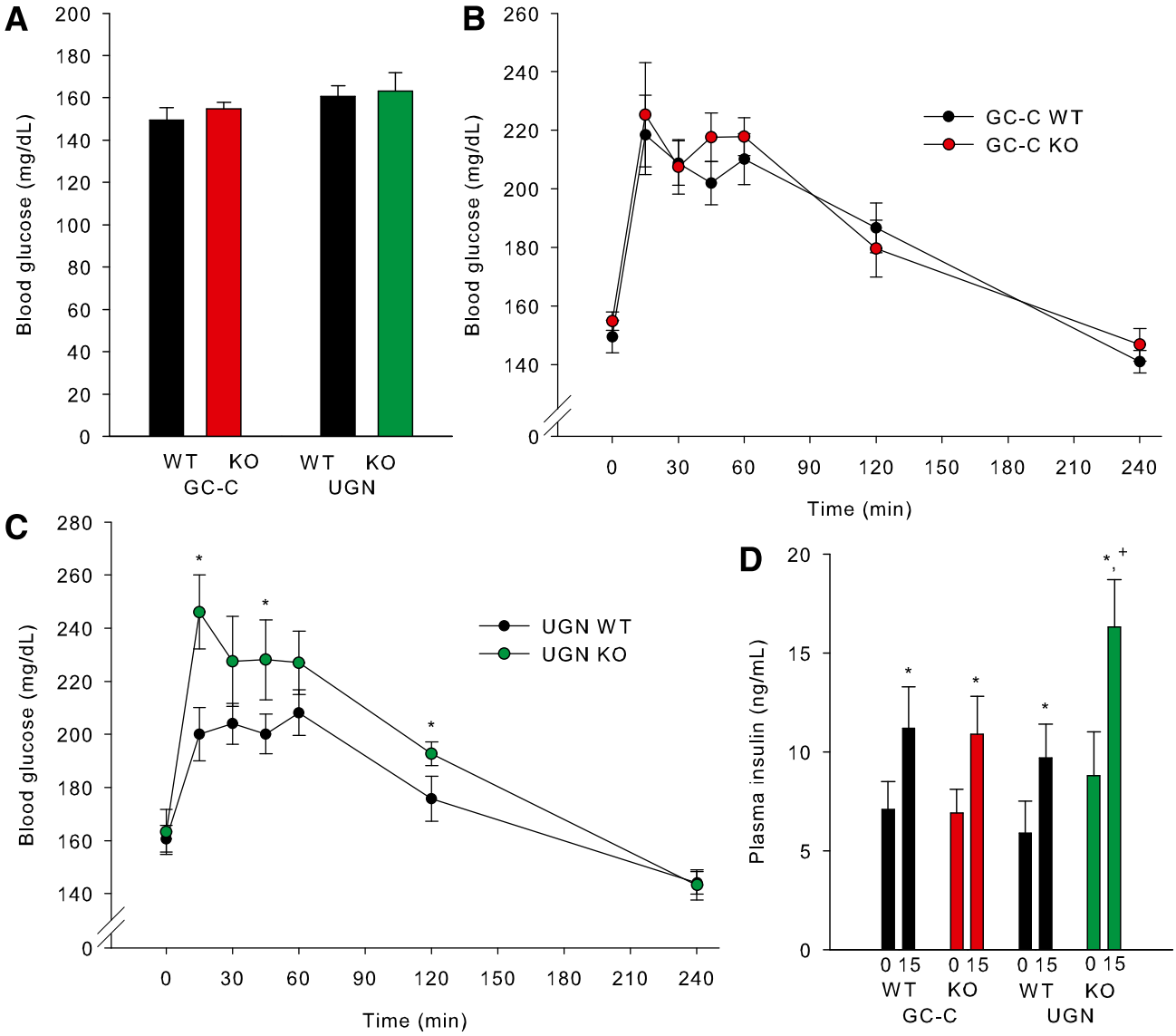

Figure 3—GC-C–deficient mice have normal tolerance to a mixed meal, whereas UGN-deficient mice have significant insulin resistance. Fasting blood glucose was not altered in either genotype (*A*). Response to a mixed-meal gavage was not different between GC-C–deficient and wild-type littermates (*B*). In contrast, UGN-deficient mice had greater glucose excursions than wild-type mice following a mixed-meal gavage (*C*). Baseline insulin levels were not altered based on genotype; 15 min after the mixed-meal gavage, UGN-deficient mice had elevated insulin compared with wild-type littermates (*D*). *$P < 0.05$ relative to WT control (*C*). *$P < 0.05$ relative to time 0; +$P < 0.05$ relative to WT control (*D*). KO, knockout; WT, wild type.

body weight in the rat. Despite this, we determined that GC-C mRNA is present in the hypothalamus, as has previously been reported (15), and that hypothalamic tissue is sensitive to I3VT-administered uroguanylin and GC-C, at least with regard to cGMP production. Together these data indicate that whereas GC-C is present and can be activated in the hypothalamus, its activation is unrelated to the short-term regulation of food intake or body weight.

Additionally, we observed no effect of GC-C knockout on any measure of metabolic status on either a chow diet or during maintenance on a HFD and development of diet-induced obesity, with GC-C$^{-/-}$ mice maintaining body weight, body composition, and food intake at the same levels as occurred in GC-C$^{+/+}$ mice. This is in contrast to a previous report where GC-C knockout mice were found to be prone to diet-induced obesity (15). Both glucose tolerance and plasma insulin levels were also unaffected by GC-C genotype in the current study.

When maintained on a chow diet, uroguanylin-deficient mice had similar body weight and composition as their wild-type littermates. However, when fed an HFD, mice lacking uroguanylin had a modest increase in body weight and body fat that was the result of a modest hyperphagia relative to their wild-type littermates. When challenged with an oral mixed meal, uroguanylin-deficient mice also displayed reduced glucose tolerance, with greater increases in blood glucose that were sustained

for a longer period. This was coupled with elevated postprandial plasma insulin, as would be predicted by the increased adiposity of the uroguanylin-deficient mice.

Interactions between diet and intestinal microflora impact energy balance, and intestinal bacterial can increase the efficiency of caloric harvesting and the level of fat deposition in adipose and liver (21). The outgrowth of obesity-prone commensals may be especially problematic when coupled with intestinal barrier dysfunction, as the enhanced release of bacterial components into the portal circulation drives the development of insulin resistance, fatty liver disease, and the low-grade systemic inflammation associated with obesity (22). Importantly, GC-C loss of function is associated with gut microflora dysbiosis and intestinal barrier dysfunction (7,23,24). Further work will be necessary to determine the potentially differential responses of GC-C$^{-/-}$ versus UGN$^{-/-}$ mice to HFD with respect to gut commensal outgrowth and release of bacterial components into the circulation. It remains possible that uroguanylin impacts body weight and insulin sensitivity via multiple mechanisms.

The disparities between our data and the report of Valentino et al. (15) are not easily explained. In part, methodological differences may have contributed to the conflicting data. For example, we allowed the mice to mature on low-fat chow prior to HFD feeding, our report focused only on male animals as opposed to a mixture of males and females, and, finally, our GC-C agonist studies were performed in rats, not mice. Despite these methodological variations, based on the differences we have observed between GC-C$^{-/-}$ and UGN$^{-/-}$ animals, it appears that the effects of uroguanylin of energy balance are independent of the GC-C receptor.

Collectively, our data suggest that uroguanylin deficiency produces modest effects on both energy and glucose homeostasis. However, the data further indicate that these effects are not mediated by the GC-C receptor. In the limited evaluation of humans with either loss or gain of GC-C–receptor function, either body weight has not been reported or no difference was observed (5,25). Collectively, our data suggest that the relationship between uroguanylin and energy homeostasis is more complicated than has been previously suggested (15) and may not be a viable target for therapeutic intervention.

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Author Contributions. D.P.B. designed the experiments, collected and analyzed the data, and wrote the manuscript. K.A.S. designed the experiments, collected and analyzed the data, provided essential reagents and analytical tools, and wrote the manuscript. J.D.M., A.P.C., R.K., and A.H. collected and analyzed the data. M.B.C. provided essential reagents and analytical tools. S.C.W. wrote the manuscript. R.J.S. designed the experiments and wrote the manuscript. All authors reviewed and edited the manuscript. D.P.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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