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Evidence That in Uncontrolled Diabetes, Hyperglucagonemia Is Required for Ketosis but Not for Increased Hepatic Glucose Production or Hyperglycemia

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Several lines of evidence implicate excess glucagon secretion in the elevated rates of hepatic glucose production (HGP), hyperglycemia, and ketosis characteristic of uncontrolled insulin-deficient diabetes (uDM), but whether hyperglucagonemia is required for hyperglycemia in this setting is unknown. To address this question, adult male Wistar rats received either streptozotocin (STZ) to induce uDM (STZ-DM) or vehicle and remained nondiabetic. Four days later, animals received daily subcutaneous injections of either the synthetic GLP-1 receptor agonist liraglutide in a dose-escalating regimen to reverse hyperglucagonemia or its vehicle for 10 days. As expected, plasma glucagon levels were elevated in STZ-DM rats, and although liraglutide treatment lowered glucagon levels to those of nondiabetic controls, it failed to attenuate diabetic hyperglycemia, elevated rates of glucose appearance (Ra), or increased hepatic gluconeogenic gene expression. In contrast, it markedly reduced levels of both plasma ketone bodies and hepatic expression of the rate-limiting enzyme involved in ketone body production. To independently confirm this finding, in a separate study, treatment of STZ-DM rats with a glucagon-neutralizing antibody was sufficient to potently lower plasma ketone bodies but failed to normalize elevated levels of either blood glucose or Ra. These data suggest that in rats with uDM, hyperglucagonemia is required for ketosis but not for increased HGP or hyperglycemia.

Several lines of evidence suggest that glucagon contributes to diabetic hyperglycemia in uncontrolled insulin-deficient diabetes (uDM). For one, hyperglucagonemia is a hallmark of type 1 diabetes (T1D) in both humans and rodent models (1,2) and is thought to drive hyperglycemia by activating hepatic gluconeogenic genes and stimulating hepatic glucose production (HGP) (3). Moreover, recent evidence that glucagon receptor (GcgR)–null mice fail to develop streptozotocin-induced diabetes (STZ-DM) suggests that glucagon signaling is required for diabetes to develop (4), with the liver playing a key role, since the protective effect is reversed after liver-specific GcgR reactivation (5). Similarly, suppressing glucagon with somatostatin during a pancreatic clamp lowers HGP (6,7), and the antidiabetic effect of leptin in a rodent model of uDM is accompanied by normalization of increased plasma glucagon levels (8). Collectively, these data support the hypothesis that hyperglucagonemia plays a key role in driving diabetic hyperglycemia and consequently that suppressing glucagon is of therapeutic value in diabetes treatment (3).

A parallel line of study has shown that leptin action in the brain normalizes blood glucose levels in uDM via an insulin-independent mechanism characterized by a normalization of both HGP and elevated plasma glucagon levels (9,10). These observations raise the possibility that the glucose-lowering action of central leptin involves normalizing glucagon levels, and conversely, that hyperglucagonemia contributes to increased HGP in uDM and other states of severe leptin and insulin deficiency. The current study was undertaken to determine if increased glucagon signaling is required for diabetic hyperglycemia. To address this question, we used complementary approaches

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to *1)* inhibit glucagon secretion and thereby reverse hyperglucagonemia and *2)* neutralize circulating glucagon. The first of these goals was accomplished by treating STZ-DM rats with the synthetic GLP-1 receptor agonist liraglutide, an agent that in addition to stimulating insulin secretion potently inhibits glucagon secretion in both rodents and humans (11,12). The second goal was achieved by treating STZ-DM rats with a glucagon-neutralizing antibody at a dose that effectively blocks glucagon-induced hyperglycemia in nondiabetic animals (13,14). Based on previous observations, we hypothesized that normalizing hyperglucagonemia would reverse diabetic ketosis, lower increased rates of HGP, and attenuate diabetic hyperglycemia in a rodent model of uDM.

RESEARCH DESIGN AND METHODS

Animals

Adult male Wistar rats (Harlan, Indianapolis, IN) were individually housed in a temperature-controlled room with a 12/12-h light/dark cycle under specific pathogen–free conditions and had ad libitum access to water and chow (PMI Nutrition, Brentwood, MO) unless otherwise stated. All procedures were performed in accordance with National Institutes of Health (NIH) Guidelines for Care and Use of Animals and were approved by the Animal Care Committee at the University of Washington.

Effect on Diabetic Hyperglycemia of Normalizing Hyperglucagonemia by Liraglutide

Adult male Wistar rats received either two consecutive daily subcutaneous injections of STZ (40 mg/kg/body weight) to induce uDM or vehicle (NaCit, pH 4.5) and remained nondiabetic. Four days after STZ-DM, animals received daily subcutaneous injections of either the synthetic GLP-1 receptor agonist liraglutide (LG; Novo Nordisk, Aalborg Øst, Denmark) or its vehicle (PBS, pH 7.4; Life Technologies, Grand Island, NY) in a dose-escalating manner up to either 300 or 500 μg/kg based on previous studies (11) as described below: LG (500 μg/kg) 25, 50, 75, 100, 150, 200, 250, 300, 400, and 500 or LG (300 μg/kg) 25, 25, 50, 75, 100, 125, 150, 200, 250, and 300. Food intake, body weight, and blood glucose levels were measured daily in the fed state. To compare the effects of liraglutide to those of insulin treatment, a separate cohort of rats was implanted subcutaneously with either an insulin pellet (2 units/day; LinShin, Toronto, Canada) or placebo 4 days after STZ-DM.

Effect on Diabetic Hyperglycemia of Blocking Glucagon Action With a Glucagon-Neutralizing Antibody

To determine if glucagon immunoneutralization blocks glucagon action, as previously reported (13–15), 4 h–fasted nondiabetic adult male Wistar rats received an intraperitoneal injection of either the glucagon-neutralizing monoclonal antibody (Glu-mAb, 4 mg/kg; Novo Nordisk) or control antibody (Con-mAb). Fifteen minutes later, all animals received a single intraperitoneal injection of glucagon (2.86 nmol/kg). To determine whether the same approach to immunoneutralization of endogenous glucagon attenuates diabetic hyperglycemia, adult male Wistar rats bearing arterial and venous catheters received either two consecutive daily subcutaneous injections of STZ (40 mg/kg/body weight) to induce uDM or vehicle (NaCit, pH 4.5) to remain nondiabetic. Four days later, STZ-DM animals received daily subcutaneous injections of either Glu-mAb (4 mg/kg) or Con-mAb for a total of 7 days. Body weight and food intake were measured daily and blood glucose was measured daily both in the fed state and 4 h after mAb injections. Eleven days after administration of STZ or vehicle, tracer dilution techniques were used to determine the effects on rates of glucose appearance (Ra) as described below.

Quantitation of Ra In Vivo

Separate cohorts of adult male Wistar rats bearing arterial and venous catheters were studied using the protocols described above for the administration of *1)* liraglutide and *2)* the glucagon-neutralizing antibody. Animals were then subject to a basal clamp in combination with tracer dilution techniques to measure Ra using [3-^{3}H]glucose (10). In brief, 4 h–fasted animals received a 24 μCi prime of [3-^{3}H]glucose at t = 0 min for 3 min followed by a continuous 0.2 μCi/min infusion for 90 min. Blood samples were taken at 10-min intervals from 60 to 90 min and processed to determine plasma [3-^{3}H]glucose as previously described (10).

Blood Collection and Assay

Plasma for [3-^{3}H]glucose determinations were deproteinized with $Ba(OH)_2$ and $ZnSO_4$ and then dried overnight at 60°C. At study completion, liver tissue was rapidly excised, snap frozen, and stored at −80°C for subsequent analysis. Blood samples were collected after 2-h food deprivation in appropriately treated tubes, as described previously (10), and centrifuged, and the plasma was removed, aliquoted, and stored at −80°C. Plasma and urinary glucose levels were measured using a GM9D Glucose Direct Analyzer (Analox Instruments, London, U.K.). Daily blood glucose levels were measured using a hand held glucometer (Accu-Chek, Corydon, IN). Plasma immunoreactive insulin and leptin levels (Crystal Chem, Downers Grove, IL) and corticosterone levels (ALPCO, Salem, NH) were determined by ELISA, 3-hydroxybutyrate (3-HB) and total ketone bodies (3-HB and acetoacetate [AcAc]) using a colorimetric kit (Wako Chemicals, Richmond, VA), and plasma glucagon levels using a glucagon RIA Kit (Linco Research, St. Charles, MO).

RT-PCR

Total RNA was extracted from the liver using TRIzol B according to the manufacturer's instructions (MRC, London, OH). Quantification of RNA was determined using spectrophotometry at 260 nm (NanoDrop 1000, Wilmington, DE) and was subsequently reverse transcribed with AMV reverse transcriptase (1 μg) (Promega, Fitchburg, WI), and real-time PCR was performed on an

ABI Prism 7900 HT (Applied Biosystems, Grand Island, NY) and analyzed as previously described (10).

Body Composition Analysis

Measures of total fat mass and lean body mass were determined using quantitative MRS (Echo Medical Systems, Houston, TX) using the Energy Balance and Glucose Metabolism Core of the Nutrition Obesity Research Center at the University of Washington.

Statistical Analysis

All results are expressed as mean ± SEM. Statistical analyses were performed using Statistica (version 7.1; StatSoft, Tulsa, OK). One-way ANOVA followed by least significant difference post hoc testing was used to test differences between more than two groups, and independent samples Student *t* tests were used for two-group comparisons. A regression method described in Zar (16) was used to test the reliability of differences in glucose or Ra between diabetic and nondiabetic control rats when each outcome was evaluated at the respective mean control value of glucagon. This method does not depend on the significance of individual group regression lines. Significance was established at $P < 0.05$, two tailed.

RESULTS

Effect of Normalizing Hyperglucagonemia by Liraglutide on Diabetic Hyperglycemia and Ketosis in STZ-DM Rats

After confirming previous evidence (11) that in nondiabetic rats, liraglutide reduces blood glucose, food intake, and body weight (data not shown), we investigated its effects in rats with STZ-DM. As expected, plasma insulin and leptin levels were markedly reduced in all STZ-DM animals relative to nondiabetic controls (Fig. 1*A*), verifying that uDM is characterized by marked deficiencies of both hormones (17). Moreover, insulin and pancreatic prepro insulin mRNA levels (data not shown) were similarly reduced in STZ-DM animals that received liraglutide as with its vehicle. As liraglutide is a potent insulin secretogogue, these data reinforce the severe insulin deficiency

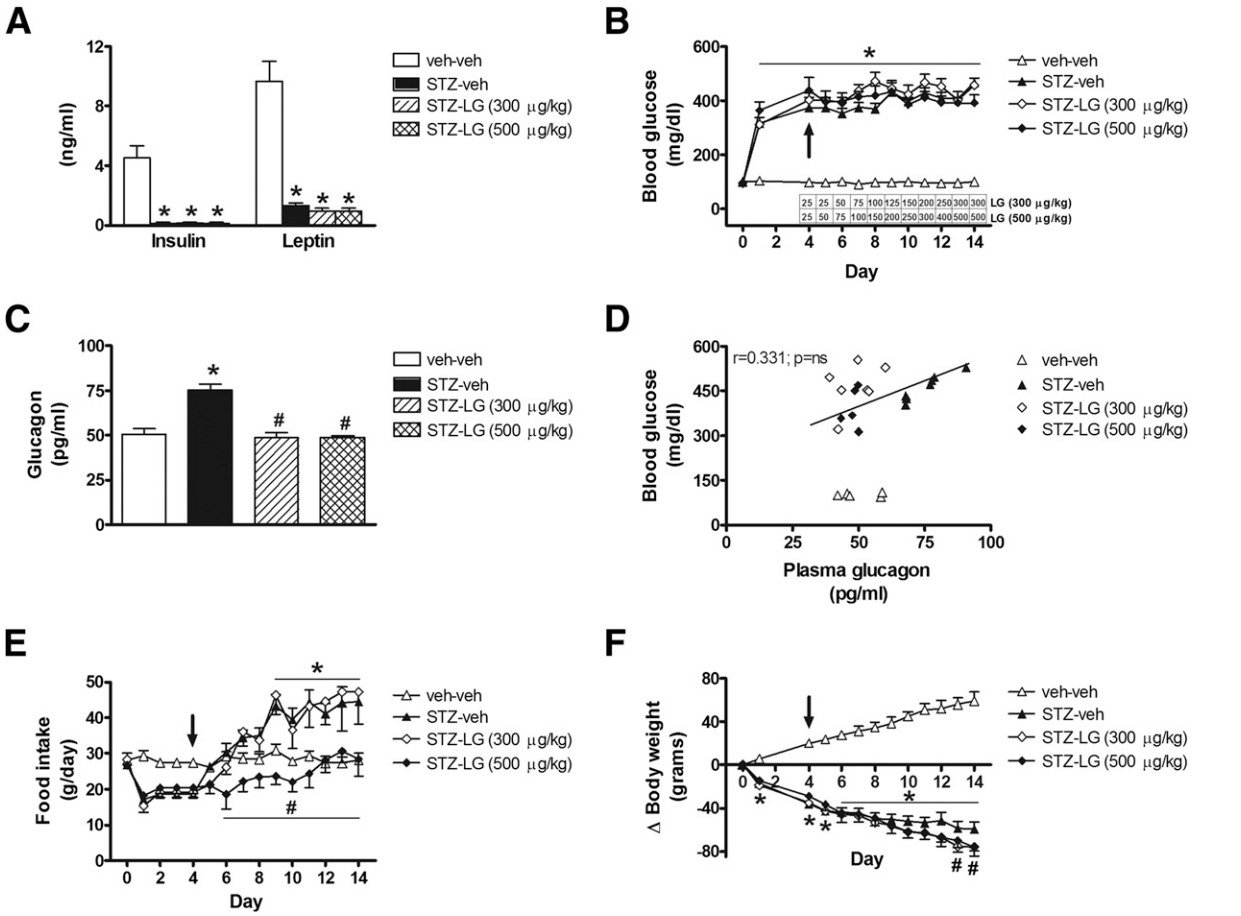

Figure 1—Normalization of hyperglucagonemia with GLP-1 receptor agonist liraglutide is not sufficient to ameliorate hyperglycemia in uDM. Plasma insulin and leptin (*A*), fed blood glucose levels (*B*), plasma glucagon levels (*C*), correlations between blood glucose and plasma glucagon levels (*D*), mean daily food intake (*E*), and body weight change (*F*) in nondiabetic controls (veh-veh) or in STZ-induced diabetic animals receiving subcutaneous injections of either vehicle (PBS, pH 7.4; STZ-veh) or the synthetic GLP-1 receptor agonist liraglutide (STZ-LG), in an escalating dose manner up to either 300 or 500 μg/kg (n = 8–10 per group). Arrow indicates the start of daily subcutaneous injections of liraglutide. Data represent mean ± SEM. *$P < 0.05$ vs. veh-veh; #$P < 0.05$ vs. STZ-veh.

characteristic of the STZ-DM model and argue against the possibility that liraglutide stimulates residual pancreatic β-cells in this setting.

As expected, STZ-vehicle–treated animals remained hyperglycemic throughout the duration of the study. However, liraglutide treatment failed to attenuate diabetic hyperglycemia relative to STZ-vehicle controls, even at doses up to 500 μg/kg (Fig. 1*B*). In contrast, liraglutide treatment effectively restored elevated plasma glucagon levels to values observed in nondiabetic controls (Fig. 1*C*). Moreover, there was no correlation between levels of glucose and glucagon among diabetic groups (Fig. 1*D*). In addition, using a biostatistical modeling approach (16), blood glucose levels remained markedly elevated in STZ-DM animals even after glucagon levels were adjusted to the level observed in nondiabetic animals (differences = 327.4 ± 29.9 mg/dL; T_{21} = 10.94; $P < 0.001$). These data indicate that *1*) the inhibitory effect of liraglutide on glucagon secretion is preserved in the setting of STZ-DM, but *2*) reversal of hyperglucagonemia by liraglutide is insufficient to ameliorate hyperglycemia in uDM.

STZ-DM animals that received vehicle exhibited the expected increase of food intake relative to nondiabetic controls, commonly referred to as "diabetic hyperphagia" (18). Whereas there was no effect of liraglutide on food intake at doses up to 300 μg/kg, diabetic hyperphagia was prevented in STZ-DM animals treated with the higher dose (500 μg/kg) (Fig. 1*E*). As expected, STZ-vehicle rats lost body weight relative to nondiabetic controls (Fig. 1*F*), despite an increase of food intake, presumably from the combined effects of glycosuria and the inability to store calories as fat induced by severe insulin deficiency. In addition, STZ-DM animals treated with liraglutide at a dose that reduced food intake consequently lost more weight than STZ-vehicle–treated controls (Fig. 1*F*). In a separate cohort of animals, we demonstrated that this effect reflects greater loss of body fat content without differences of lean body mass (Fig. 2*A* and *B*).

Although the marked glycosuria characteristic of uDM was not prevented in animals treated with liraglutide, the increase of water intake characteristic of STZ-DM was moderately reduced, consistent with previous reports of liraglutide to reduce water consumption in nondiabetic animals (19) (Fig. 2*C* and *D*). Moreover, although liraglutide treatment failed to attenuate the increase of plasma corticosterone levels characteristic of STZ-DM, the increase of either total plasma ketone bodies or 3-HB alone characteristic of STZ-DM was significantly blunted by liraglutide (Fig. 2*E–G*), consistent with the hypothesis that hyperglucagonemia is required for increased ketone body production in this setting (20,21). In further support of this hypothesis, plasma glucagon levels were strongly predictive of plasma ketone body levels across all groups ($r = 0.681$; $P < 0.001$) (Fig. 2*H*).

Effect of Liraglutide on HGP in STZ-DM Rats

Our finding that liraglutide normalized elevated glucagon levels without attenuating diabetic hyperglycemia raises the question of whether increased HGP in STZ-DM is dependent on hyperglucagonemia. To answer this question, we used tracer dilution techniques during a basal clamp (e.g., without insulin or glucose infusion) to determine whether liraglutide-induced normalization of glucagon levels reduces Ra in STZ-DM rats. Consistent with previous reports and the known role played by increased HGP in diabetic hyperglycemia (10), we found that Ra was markedly elevated in STZ-vehicle–treated animals relative to nondiabetic controls. However, liraglutide treatment at a dose sufficient to suppress hyperglucagonemia failed to significantly lower elevated rates of Ra (Fig. 3*A–C*). Moreover, we found that Ra values were markedly higher in STZ-DM animals even after glucagon levels had been adjusted statistically to the levels seen in nondiabetic animals (T_{10} = 2.28; $P < 0.05$), implying that increased Ra was not causally linked to elevated circulating glucagon levels. Combined with the absence of a significant correlation between glucose and glucagon levels among STZ-DM rats, these analyses establish that variation in plasma glucagon levels cannot explain either increased Ra or hyperglycemia in uDM.

To gain additional insights into the mechanism(s) whereby suppression of glucagon lowers ketosis but not HGP, we measured hepatic expression of genes encoding the enzymes that are rate limiting for ketogenesis (mitochondrial hydroxymethyl glutaryl [HMG]–CoA synthase [*Hmgcs2*]) (22) and gluconeogenesis (*G6Pase* and *Pepck*). We found that whereas elevated hepatic expression of *G6Pase* and *Pepck*, characteristic of vehicle-treated rats with STZ-DM, was not reversed by liraglutide treatment, despite normalization of glucagon levels (Fig. 3*D* and *E*), the increased hepatic expression of *Hmgcs2* was markedly reduced in STZ-DM animals that received liraglutide (Fig. 3*F*). These biochemical data strengthen the interpretation that in rats with uDM, hyperglucagonemia plays a key role to drive hepatic ketogenesis but is dispensable for hepatic mechanisms driving elevated HGP and associated hyperglycemia.

Effect of Immunoneutralization of Endogenous Glucagon on HGP and Ketosis in STZ-DM Rats

To verify the ability of the Glu-mAb to inactivate circulating glucagon, we measured the effect of exogenous glucagon on blood glucose levels in either the presence or absence of Glu-mAb pretreatment in nondiabetic rats. As expected, we found that the glycemic effect of exogenous glucagon was completely blocked by pretreatment with the Glu-mAb (blood $glucose_{t = 20\ min}$: 148.2 ± 7.0 vs. 180.5 ± 13.7 mg/dL; $P < 0.05$). We next examined the effect of the Glu-mAb in the setting of STZ-DM and found that relative to the Con-mAb, administration of the Glu-mAb failed to lower blood glucose levels or either food or water intake, but it did attenuate body weight loss (Fig. 4*A–D*). Moreover, using tracer dilution techniques, we found that Ra was markedly elevated in STZ-DM Con-mAb–treated rats relative to nondiabetic controls, and

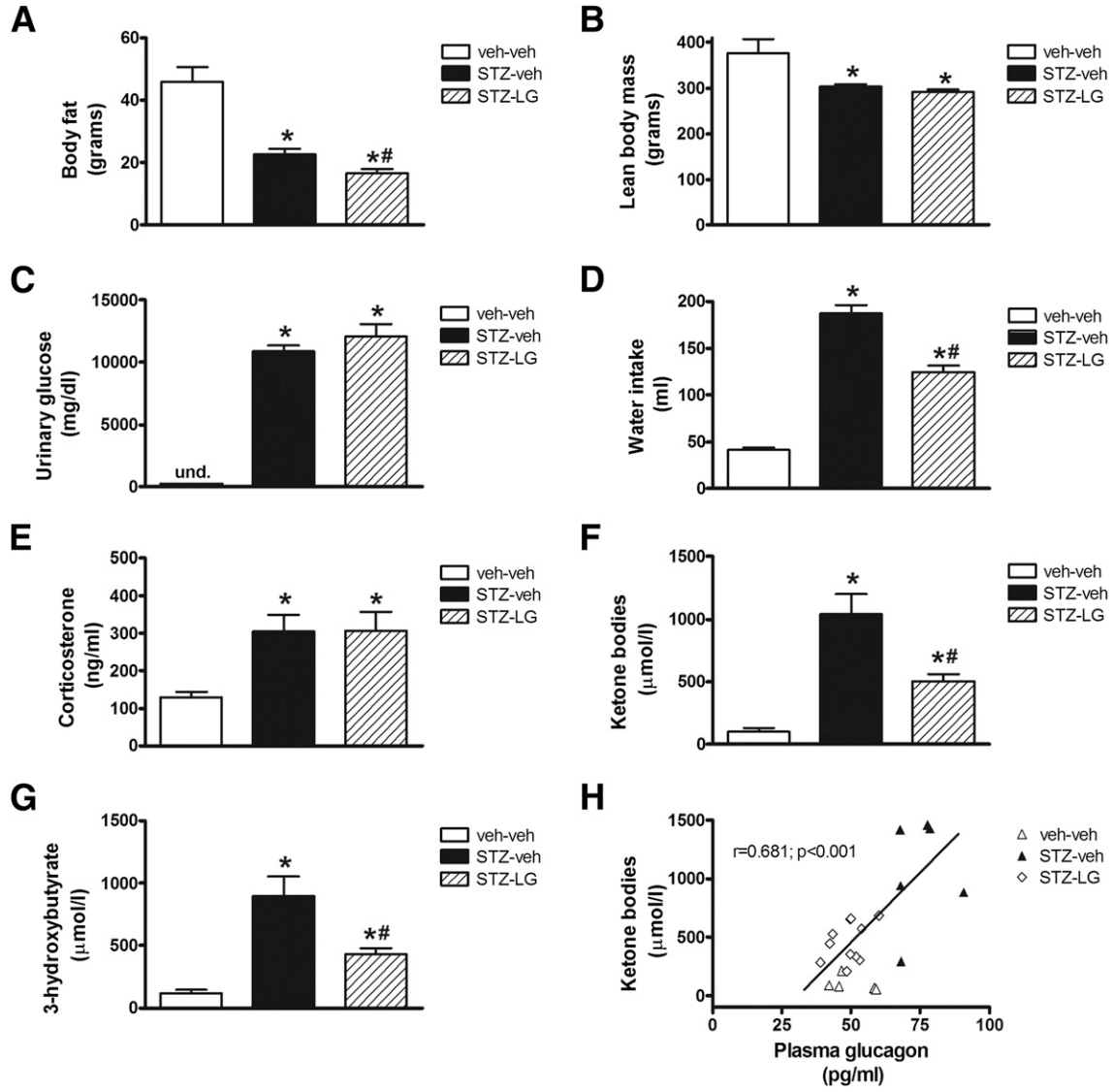

Figure 2—Normalization of hyperglucagonemia with the GLP-1 receptor agonist liraglutide attenuates ketosis in uDM. Body fat (*A*), lean body mass (*B*), urinary glucose (*C*), daily water intake (*D*), plasma corticosterone (*E*) and total ketone body levels (AcAc and 3-HB) (*F*), 3-HB levels alone (*G*), and the correlation between total ketone body levels and plasma glucagon levels (*H*) in nondiabetic controls (veh-veh) or in STZ-induced diabetic animals receiving subcutaneous injections of either vehicle (PBS, pH 7.4; STZ-veh) or the synthetic GLP-1 receptor agonist liraglutide (STZ-LG), in an escalating dose manner up to 300 μg/kg (n = 8–10 per group). Data represent mean ± SEM. *$P < 0.05$ vs. veh-veh; #$P < 0.05$ vs. STZ-veh. und., undetectable.

that this elevation of Ra was maintained in STZ-DM rats treated with Glu-mAb (Fig. 4*G* and *H*). Thus, based on data using a second independent method, we conclude that increased glucagon signaling does not appear to be required for either hyperglycemia or increased HGP in rats with uDM. By comparison, treatment of STZ-DM rats with Glu-mAb potently lowered plasma ketone body levels without effects on elevated plasma corticosterone levels (Fig. 4*E* and *F*). Taken together, these data suggest that immunoneutralization of endogenous glucagon with a glucagon-specific mAb at a dose that markedly attenuates diabetic ketosis is insufficient to ameliorate either hyperglycemia or increased Ra in uDM.

Effect of Insulin Treatment on Diabetic Hyperglycemia and Ketosis in STZ-DM Rats

We next compared the metabolic and biochemical effects of glucagon suppression alone with that of insulin treatment in the setting of uDM. As expected, treatment with a physiological replacement dose of insulin was sufficient to markedly lower blood glucose levels in STZ-DM rats (Fig. 5*A* and *B*), as well as lower elevated plasma levels of both glucagon and ketone bodies (AcAc/3-HB or 3-HB alone) to values seen in nondiabetic controls (Fig. 5*C* and *D*). These insulin effects were accompanied by normalization of elevated hepatic expression of *G6Pase* and *Pepck* as well as *Hmgcs2* (Fig. 5*E* and *F*). In addition, insulin treatment increased body weight, body adiposity, and

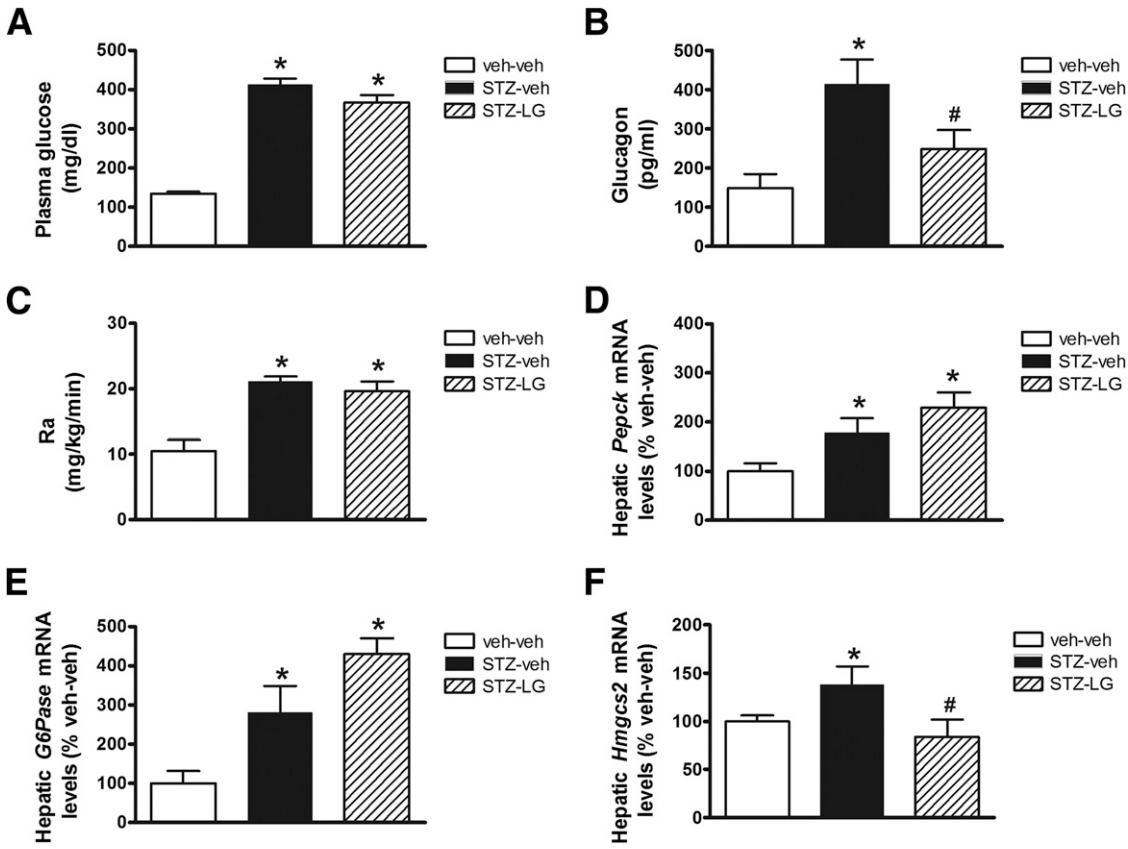

Figure 3—Normalization of hyperglucagonemia with the GLP-1 receptor agonist liraglutide fails to suppress increased Ra or hepatic gluconeogenic gene expression in uDM. Five hour–fasted plasma glucose (*A*) and glucagon levels (*B*); Ra, as determined from [3-^{3}H] glucose tracer studies during a basal clamp (*C*); as well as hepatic expression of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*Pepck*) (*D*) and glucose-6-phosphatase (*G6Pase*) (*E*) and the ketogenic gene mitochondrial HMG-CoA synthase (*Hmgcs2*) (*F*) using real-time PCR in STZ-induced diabetic animals receiving subcutaneous injections of either vehicle (PBS, pH 7.4; STZ-veh) or the synthetic GLP-1 receptor agonist liraglutide (STZ-LG), in an escalating dose manner up to 300 μg/kg (n = 6–8 per group). Data represent mean ± SEM. *P < 0.05 vs. veh-veh; #P < 0.05 vs. STZ-veh. Veh-veh, nondiabetic controls.

plasma leptin levels, while also attenuating diabetic hyperphagia (Fig. 5*G–J*). These data collectively indicate that even though excess glucagon secretion during uDM is corrected by both insulin and liraglutide, insulin has the capacity to lower gluconeogenic gene expression and blood glucose levels whereas liraglutide does not, implying that glucagon secretion and control of circulating glucose concentrations can be fundamentally uncoupled from one another in uDM.

To further evaluate this hypothesis, we compared the relationship between plasma glucagon levels with those of blood glucose, ketone bodies, and hepatic gluconeogenic and ketogenic gene expression across all nondiabetic and STZ-DM animals and superimposed on this analysis data from STZ-DM animals treated with either liraglutide or insulin. This comprehensive approach revealed a significant, positive correlation between plasma glucagon and blood glucose levels (r = 0.559; $P < 0.01$), hepatic expression of gluconeogenic (*G6Pase*: r = 0.487; $P < 0.01$; *Pepck*: r = 0.597 [data not shown]; $P < 0.01$) and ketogenic (*Hmgcs2*: r = 0.417; $P < 0.05$) genes, as well as plasma ketone body levels (r = 0.371; $P < 0.05$) (Fig. 6*A*, *C*, *E*, and *G*) when including the STZ-DM animals that received either vehicle or insulin. In contrast, however, when STZ-DM animals that received either vehicle or liraglutide were included in the analysis, there was no significant correlation between plasma glucagon and either blood glucose or hepatic gluconeogenic gene expression (P = NS), whereas the significant relationship between plasma glucagon and both ketone bodies (r = 0.318; $P < 0.05$) and hepatic *Hmgcs2* gene expression (r = 0.549; $P < 0.01$) (Fig. 6*B*, *D*, *F*, and *H*) was preserved.

DISCUSSION

Several lines of evidence implicate hyperglucagonemia in both the increased HGP and hyperglycemia characteristic of T1D (3), but whether increased glucagon signaling is required for these responses in conditions of severe insulin and leptin deficiency is unknown. In the current studies, we investigated whether reversal of hyperglucagonemia using either a GLP-1 analog or a glucagon-neutralizing antibody is sufficient to normalize HGP and lower elevated blood glucose levels in a rat model of severe uDM. Here, we report that neither systemic administration of liraglutide, which reverses hyperglucagonemia in

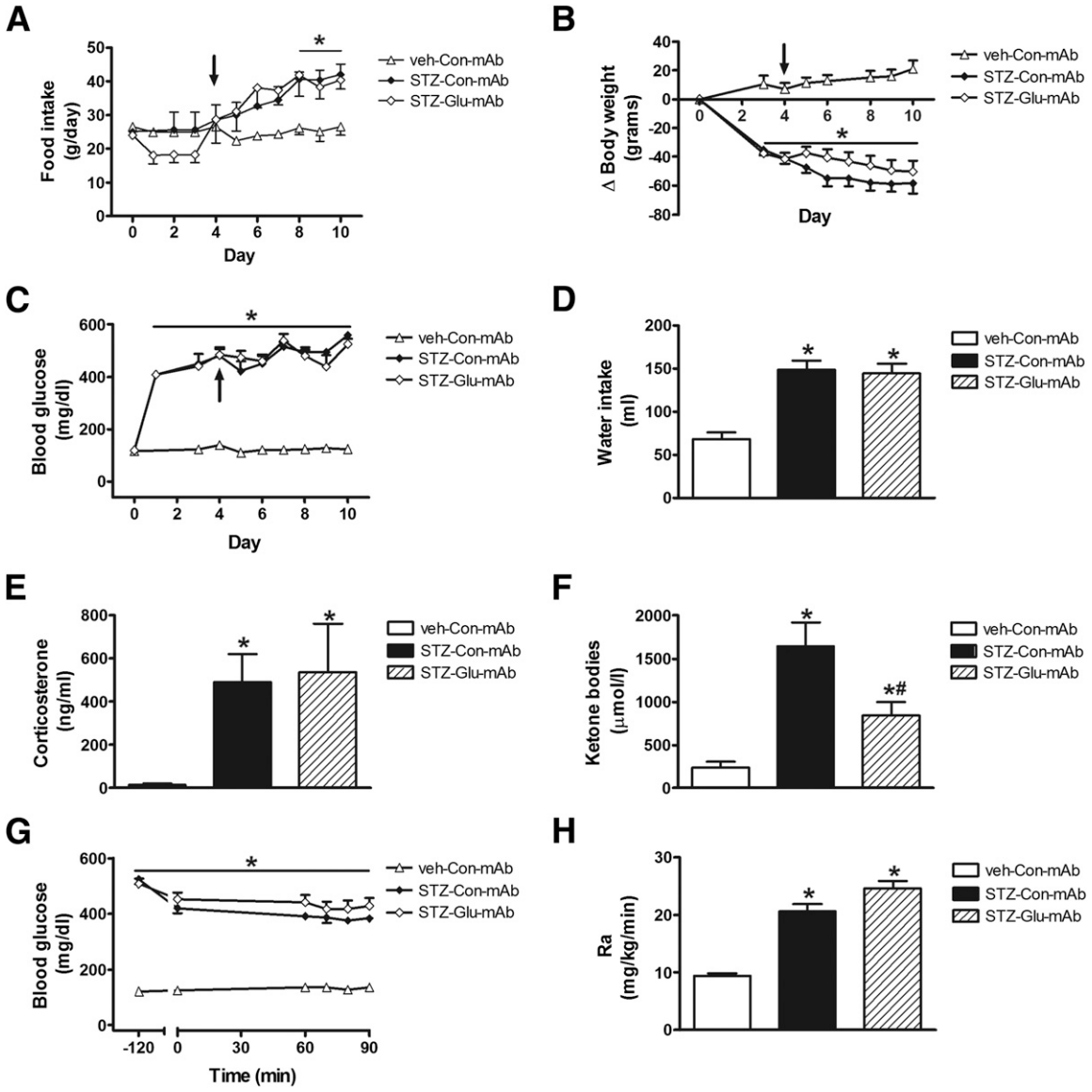

Figure 4—Immunoneutralization of endogenous glucagon fails to lower blood glucose or Ra but attenuates ketosis in uDM. Food intake (*A*), body weight change (*B*), fed blood glucose levels (*C*), water intake (*D*), plasma corticosterone (*E*), plasma ketone body levels (*F*), fasted plasma glucose levels (*G*), and Ra (*H*), as determined from [3-^{3}H]glucose tracer studies during a basal clamp in STZ-induced diabetic animals that received either daily subcutaneous injections of the glucagon-neutralizing antibody (Glu-mAb) or control antibody (Con-mAb) (n = 6–7 per group). Data represent mean ± SEM. *P < 0.05 vs. veh-Con-mAb; #P < 0.05 vs. STZ-Con-mAb.

STZ-DM rats, nor administration of a glucagon-neutralizing antibody has any impact on diabetic hyperglycemia, elevated Ra, or increased hepatic gluconeogenic gene expression characteristic of uDM. In marked contrast, both treatments were sufficient to ameliorate diabetic ketosis. Taken together, these data suggest that *1*) normalization of glucagon levels alone in uDM is insufficient to correct hyperglycemia in severe uDM and *2*) the requirement for hyperglucagonemia in diabetic ketogenesis is separable from its role to drive HGP in this setting.

Glucagon is a major positive regulator of hepatic production of both ketone bodies and glucose. Since plasma glucagon levels are elevated in uDM, it follows logically that hyperglucagonemia would play a key role in both hyperglycemia and ketosis in this setting (3,20). Consistent with this hypothesis, suppression of glucagon with infusion of somatostatin in both dogs with alloxan-induced diabetes and humans with T1D (1,3) lowers blood glucose levels and HGP and reverses diabetic ketoacidosis (20,21), and similar findings were observed when leptin was administered at pharmacological doses to STZ-DM rodents (8–10). Our new findings suggest that hyperglucagonemia is not required for either increased HGP or hyperglycemia in rats with STZ-DM. Using liraglutide to inhibit glucagon secretion in STZ-DM rats, we found that normalization of elevated plasma glucagon levels has little

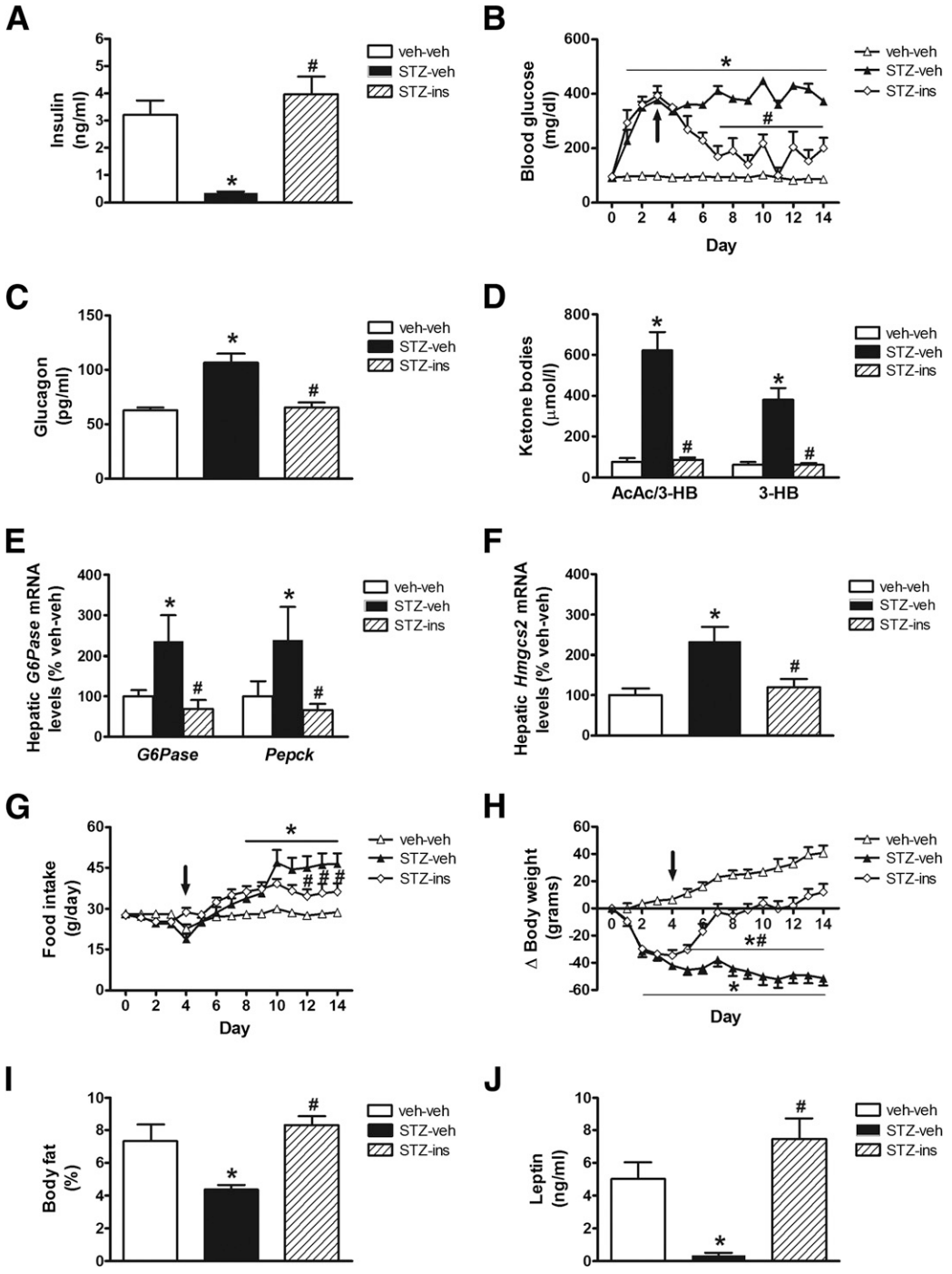

Figure 5—Insulin treatment normalizes diabetic hyperglycemia, hyperglucagonemia, and ketosis characteristic of uDM. Plasma insulin (*A*), fed blood glucose (*B*), plasma glucagon (*C*), and ketone body levels (AcAc and 3-HB and 3-HB alone) (*D*) and hepatic expression of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6Pase*) (*E*) and the ketogenic gene mitochondrial HMG-CoA synthase (*Hmgcs2*) (*F*) using real-time PCR, food intake (*G*), body weight change (*H*), percent body fat (*I*), and plasma leptin levels (*J*) in nondiabetic controls (veh-veh) or in STZ-induced diabetic animals that were implanted either with an insulin pellet (STZ-ins; 2 units/day) or placebo (STZ-veh) (n = 6–7 per group). Data represent mean ± SEM. *P < 0.05 vs. veh-veh; #P < 0.05 vs. STZ-veh.

impact on either HGP or hyperglycemia, even though it does markedly attenuate diabetic ketosis. Similarly, whereas plasma ketone levels were dramatically lowered by immunoneutralization of endogenous glucagon with a monoclonal antibody, this intervention had little effect on either HGP or glycemia. Therefore, whereas increased glucagon signaling is required for ketosis in rats with STZ-DM, this does not appear to be the case for hyperglycemia.

Previous evidence suggests that in the setting of STZ-DM, increased rates of HGP and associated hyperglycemia

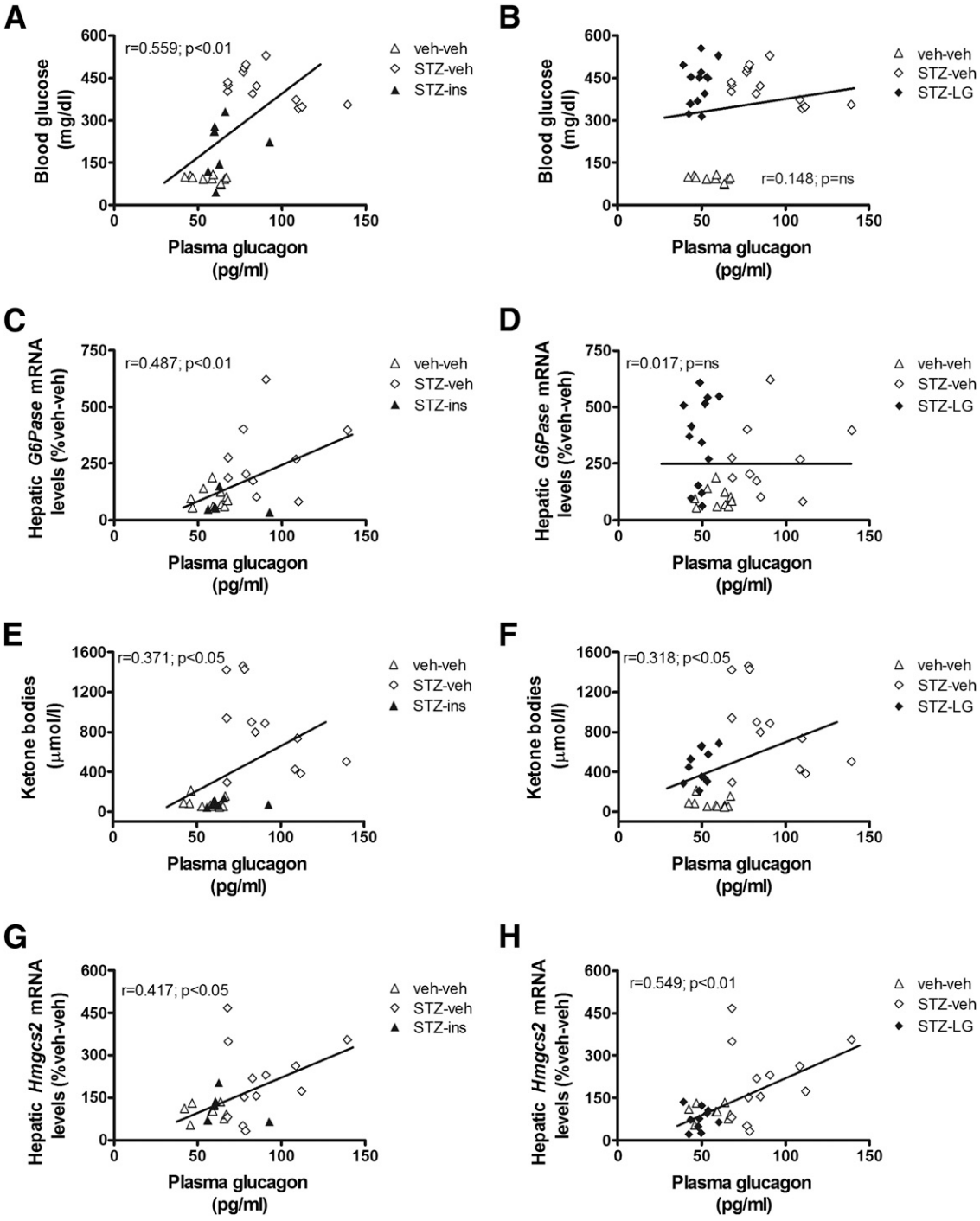

Figure 6—Effect of liraglutide and insulin on the relationship between plasma glucagon and glycemia and ketosis. Correlation between plasma glucagon and blood glucose (*A* and *B*), hepatic expression of glucose-6-phosphatase (*G6Pase*) (*C* and *D*), ketone body levels (*E* and *F*), and hepatic expression of mitochondrial HMG-CoA synthase (*Hmgcs2*) (*G* and *H*) in nondiabetic (veh-veh) and STZ-DM animals that received vehicle (STZ-veh) and STZ-DM animals that received either insulin (STZ-ins) or the synthetic GLP-1 receptor agonist liraglutide (STZ-LG).

are driven in part by increased expression of the key hepatic gluconeogenic genes, *Pepck* and *G6P*ase (23). Our findings that liraglutide normalized elevated plasma glucagon levels in STZ-DM rats, yet failed to suppress elevated expression of these gluconeogenic genes, suggest that mechanisms driving HGP in the setting of severe insulin deficiency do not require an increased glucagon signal. We interpret these findings to suggest that increased glucagon levels are one of several redundant mechanisms driving HGP in this setting. At the cellular level, GcgR activation increases cAMP levels, which in turn activates protein kinase A (PKA). Gluconeogenic

gene expression is induced by PKA through *1*) phosphorylation of cAMP response element–binding protein (CREB) (24), and *2*) dephosphorylation of the CREB-regulated transcriptional coactivator 2 (CRTC2), also known as TORC2 (25). In the setting of severe insulin deficiency, however, molecular events involving activation of the forkhead box transcription factor 1 (FoxO1) appear to drive gluconeogenesis via a mechanism that does not require increased PKA signaling. Specifically, the effect of insulin to inhibit hepatic gluconeogenesis involves activation of the IRS→PI3K→Akt pathway and subsequent phosphorylation of FoxO1 by Akt. This phosphorylation event leads to the nuclear exclusion of FoxO1 and suppression of its transcriptional activity (26). In the absence of insulin, however, FoxO1 becomes constitutively active, inducing transcription of *Pepck* and *G6Pase* in an uncontrolled manner. Based on these considerations, we hypothesize that when insulin is present at levels sufficient to inactivate FoxO1, increased glucagon signaling plays an important positive role to control HGP, but that in the setting of uDM, unrestrained FoxO1 activity effectively drives HGP without the need for increased glucagon signaling. Carefully designed and comprehensive studies will be needed to critically test this hypothesis.

In contrast to its lack of effect on HGP and glycemia, we found that liraglutide effectively attenuated ketosis in uDM and that this effect was accompanied by suppression of the rate-limiting enzyme involved in ketone body synthesis, HMGCS2. Glucagon is a key driver of ketogenesis (20,27), and biochemical evidence suggests that glucagon activates HMGCS2 to promote ketone body production (28). This effect involves glucagon-mediated activation of the forkhead box transcription factor Foxa2 (via p300 acetylation), which binds to the *Hmgcs2* promoter and activates its transcription (29,30). In addition, glucagon activates mitochondrial HMGCS2 post-translationally by decreasing succinylation of the enzyme (31), and our new data suggest that in uDM, these glucagon-mediated effects help to drive ketogenesis.

We interpret these findings to suggest that in established uDM, hyperglucagonemia is one of several redundant mechanisms that drive increased HGP and hyperglycemia. With such redundancy, it may be that none of these mechanisms is individually required for hyperglycemia to occur in the setting of severe insulin and leptin deficiency. This interpretation is consistent with the established role of glucagon in the counterregulatory response to hypoglycemia; increased secretion of glucagon clearly plays a role, but because multiple, redundant mechanisms contribute, selective loss of the contribution made by elevated glucagon levels does not compromise recovery from hypoglycemia (32). This perspective also informs our interpretation of discrepancies between our findings and previous observations. For example, glucose-lowering effects of somatostatin and leptin likely involve mechanisms in addition to inhibition of glucagon secretion (5). Also, the degree to which diabetes is truly "uncontrolled" (e.g., associated with absolute deficiency of both insulin and leptin) may play an important role. Thus, whether elevated glucagon levels worsen glycemia may depend on the severity of the insulin deficiency, as described above.

Consistent with this view and our current observations, previous studies have demonstrated that Glu-mAb fails to lower blood glucose levels in severely hyperglycemic (and insulin deficient) STZ-DM rats (14). In STZ-DM rats characterized by hyperglycemia and more moderate insulin deficiency (14), or when STZ-DM rats or rabbits are treated with insulin (14,15), Glu-mAb treatment does have a glucose-lowering effect. These findings collectively suggest that when insulin is present in sufficient amounts, fewer redundant mechanisms are recruited to drive hyperglycemia, and glucagon neutralization therefore elicits detectable glucose-lowering effects. Conversely, in euglycemic clamp studies in patients with T1D, glucagon markedly increases endogenous glucose production at lower infusion rates of insulin, but this effect is either attenuated or abolished as insulin levels are raised (33). Thus, it is possible that the concentration of glucagon that has a maximal effect on glucose production decreases as insulin concentrations fall.

We stress that our study was intended simply to test whether normalization of hyperglucagonemia to nondiabetic control levels ameliorates hyperglycemia and hepatic responses to uDM, which is different from asking whether glucagon signaling per se is required. Indeed, data from GcgR-deficient mice suggest that glucagon signaling is required for diabetes pathogenesis (4,5), but efforts to generalize such findings to genetically intact animals should be made with caution, since both basal and stimulated glucagon action are abolished in this model. Moreover, GcgR-deficient mice exhibit a profound lean, hypermetabolic phenotype characterized by fasting hypoglycemia and marked elevations in circulating GLP-1 (34) and FGF21 levels (35), and recent evidence suggests that both hormones help to ameliorate diabetic hyperglycemia in mice lacking GcgR (35). In contrast, administration of either leptin or liraglutide can restore glucagon levels to normal in uDM, but they may not elicit the unintended consequences of increased secretion of other, seemingly unrelated glucoregulatory hormones.

In addition to severe insulin deficiency, uDM is also characterized by profound leptin deficiency in rodent models (17). Indeed, the ability of insulin treatment to reverse uDM likely involves normalization of plasma leptin levels (17), since leptin deficiency causes severe insulin resistance and is itself diabetogenic. Moreover, recent evidence suggests that leptin action in the brain normalizes blood glucose levels in uDM, in part by normalizing HGP, an affect accompanied by normalization of plasma glucagon levels (9,10). In uDM, therefore, central leptin deficiency may contribute to elevated levels of both glucagon and HGP. In this context, it is noteworthy that insulin treatment of rats with STZ-DM normalized both elevated

levels of both glucose and glucagon, while also restoring low insulin and leptin levels to normal (17). These observations raise the possibility that combined insulin and leptin deficiency drives increased HGP and that hyperglucagonemia is one of several redundant mechanisms that underlie this response. Consistent with this hypothesis, physiological leptin replacement in STZ-DM rats is sufficient to normalize plasma glucagon levels but has little effect on diabetic hyperglycemia (36). One additional mechanism that may contribute to diabetic hyperglycemia in the presence of normal plasma glucagon levels in uDM is increased glucocorticoid secretion, since plasma corticosterone levels remained elevated in STZ-DM rats treated with liraglutide and Glu-mAb, and recent evidence suggests that glucocorticoid-induced lipolysis plays a key role to drive hyperglycemia in fasted STZ-DM rats at the onset of insulin deficiency (37). However, previous evidence suggests that hypercorticosteronemia is not required for diabetic hyperglycemia since adrenalectomy does not prevent hyperglycemia in rats with established STZ-DM (38) and the suppression of elevated corticosterone levels is not required for leptin's antidiabetic effects since, like glucagon, physiological leptin replacement normalizes plasma corticosterone levels in STZ-DM rats but does not ameliorate hyperglycemia (36).

As potent insulin secretogogues, GLP-1 analogs have an established niche in the treatment of type 2 diabetes (T2D) (39). In individuals with T1D, however, this effect is absent, since most of these patients lack significant insulin secretory capacity. Previous evidence suggests that adjunctive therapy with GLP-1 analogs in individuals with T1D suppresses glucagon levels, and this effect reduces insulin requirements with improved or unaltered glycemic control (40,41) and lowers postprandial hyperglycemia (41). Our new findings raise the possibility that the glucagon-lowering action of GLP-1 analogs could also be of benefit in the treatment of ketoacidosis in humans with either T1D or T2D. In addition, our findings also show that the effect of increased GLP-1 receptor signaling to promote negative energy balance (42) is preserved in the setting of uDM. Consistent with observations in rodents and humans (11,43), we found that liraglutide treatment reduced food intake, body weight, and body adiposity in STZ-DM rats. These findings raise the possibility that GLP-1 receptor analogs may also offer a useful adjunct to insulin in the treatment of T1D by limiting weight gain (44).

In summary, these findings suggest that lowering elevated glucagon levels into the normal range does not improve glucose production or blood glucose levels in the setting of uDM. However, these data also suggest that treatment with GLP-1 receptor analogs or GcgR antagonists alone in T1D will have a beneficial effect to attenuate ketosis. Future studies to assess these effects in humans with T1D are warranted, as well as to determine if GLP-1 analogs can be used effectively as adjuncts to insulin in this setting (45).

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