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Effects of glucose and insulin on acyl ghrelin and desacyl ghrelin, leptin, and adiponectin in pregnant women with diabetes

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Abstract

The aim of the study was to compare the regulation of ghrelin, leptin, and adiponectin by insulin and glucose during the second and third trimesters of pregnancy in women with diabetes. We studied 9 pregnant women with diabetes. All women were treated with insulin and omitted the morning dose on the day of the test. After collection of baseline fasting samples, we performed 3 successive glucose clamps: 2 euglycemic clamps (glucose, 5 mmol/L; insulin infusion at 20 and 40 mU m^{-2} min^{-1}) and 1 hyperglycemic clamp (glucose, 10 mmol/L; insulin infusion at 40 mU m^{-2} min^{-1}). We determined concentrations of acyl and desacyl ghrelin (using a double-antibody sandwich assay that recognizes the full-length molecule), leptin, and adiponectin. Fasting desacyl ghrelin concentrations decreased, whereas insulin and leptin concentrations increased, between the second and third trimesters of pregnancy ($P \leq .011$). During the clamp studies, desacyl ghrelin concentrations decreased by 33% (second trimester, $P = .004$) and 27% (third trimester, $P = .09$) with increasing glucose and insulin concentrations, whereas acyl ghrelin, leptin, and adiponectin concentrations were unaffected. Glucose and insulin regulate desacyl ghrelin concentrations in pregnant women with diabetes. Impaired desacyl ghrelin regulation may affect energy metabolism in pregnant women with poorly controlled diabetes.

1. Introduction

Ghrelin is a peptide hormone mainly secreted by the stomach. It circulates as acyl ghrelin (AG) and desacyl ghrelin (DG). The presence of an acyl chain (octanoate residue) added by the enzyme ghrelin-*O*-acyl transferase to the serine 3 moiety of ghrelin [1,2] is required for binding to the growth hormone secretagogue receptor, which mediates many of the actions ascribed to ghrelin [3]. Once in the plasma, AG is rapidly deacylated into DG by cholinesterase [4,5].

Both AG and DG potentially play a role in energy balance. Exogenous administration of AG stimulates GH secretion [6] and has orexigenic effects [7] in humans. Although the role of DG in control of food intake is controversial [8,9], in rats, both AG and DG promote adipogenesis via growth hormone secretagogue receptor–independent pathways that remain to be characterized [10]. Recent in vitro evidence suggests this may also be true in humans [11]. Ghrelin concentrations increase with fasting and decrease after caloric intake [12-14], suggesting a role in hunger and meal initiation. Ghrelin concentrations are also closely linked to glucose metabolism. Hyperglycemia and insulin resistance [15,16] are independently associated with a decrease in ghrelin concentrations [17].

In humans, we recently characterized ghrelin homeostasis in pregnant women with and without gestational diabetes [5]. We found markedly decreased AG concentrations during pregnancy (the significance of which remains unclear), whereas DG concentrations were similar during pregnancy

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and after delivery. The expected decrease in both AG and DG after ingestion of a meal was preserved during pregnancy. Surprisingly, despite higher glucose concentrations, gestational diabetes did not affect AG concentrations. Glucose tolerance was only mildly affected in these women, whose gestational diabetes was successfully controlled with diet alone. In contrast, DG concentrations were almost doubled in subjects with gestational diabetes compared with control subjects, not only during pregnancy but also during the postpartum period.

We hypothesized that pregnant subjects with preexisting diabetes may be more insulin resistant and that acute changes in glucose and insulin concentrations would affect AG and DG concentrations. The objective of the current study was to compare AG and DG concentrations at 2 time points during diabetic pregnancy using a series of glucose and insulin clamps. We also investigated the relationship between ghrelin, leptin, and adiponectin under these experimental conditions.

2. Methods

Nine pregnant women with diabetes were recruited from the Diabetes and Pregnancy Clinic at Children's and Women's Health Centre (DT). The subjects were part of a research protocol investigating the role of glucose and insulin on carotid artery relaxation. Eight had been diagnosed with type 2 diabetes mellitus before pregnancy according to the Canadian Diabetes Association criteria [18], and one had mature-onset diabetes of the young due to a confirmed glucokinase missense mutation (L324P, patient 10 in McKinney et al [19]). Inclusion criteria were as follows: older than 18 years, singleton pregnancy, and no preeclampsia. Before pregnancy, treatments included diet (n = 1), metformin alone (n = 4), metformin + glyburide (n = 2), or insulin (n = 2).

During pregnancy, all subjects were receiving 2 daily injections of neutral protamine Hagedorn insulin and regular insulin (before breakfast and before supper). The subjects' diabetes was well controlled on the twice-daily regimen. Detailed daily recordings of blood glucose were available for 8 subjects (1 subject, who also did not return for the second clamp study, failed to provide records). These records showed a mean glucose (±SE) before meals of 5.11 ± 0.08 mmol/L; at bedtime, the mean ± SE glucose was 5.38 ± 0.16 mmol/L (n = 8). The mean average number of weeks before delivery for which detailed recordings were available was 30.5 (median, 30.5; range, 25-36).

Each subject underwent 2 glycemic clamp studies, once during the second trimester of pregnancy (12-18 weeks) and once during the third trimester of pregnancy (30-36 weeks). Each study was composed of a succession of 3 clamps (performed over a 6-hour period) that differed by their glycemic target and their rate of insulin infusion (see below). At delivery, birthweight and gestational age were recorded; and birthweight *z* score was calculated [20]. Baseline characteristics, gestational age at delivery, and birth weight are presented in Table 1. The study protocol was approved by the University of British Columbia Clinical Research Ethics Board and by the institutional review boards of the Vancouver Coastal Health Authority and the Children's and Women's Health Centre of British Columbia. After detailed explanation of the study, all patients gave free, written informed consent to the procedures, which were conducted in accordance with Declaration of Helsinki principles.

2.1. Clamp studies

Subjects were admitted to the testing room between 7:00 and 8:00 AM after an overnight fast and omitted their morning insulin injection. Weight (in kilograms) and height (in meters) were used to calculate body surface area ([kilograms*meters/36]$^{1/2}$) and body mass index (BMI) (kilograms per square meter). An arm vein was cannulated for infusion of potassium phosphate, insulin, and dextrose; and a contralateral hand vein was cannulated in a retrograde fashion for sampling, with the hand placed in a heated box (70°C) to "arterialize" venous blood. Baseline insulin and glucose concentrations were determined at −10 and 0 minutes before insulin and glucose infusions. We then began infusing insulin at a rate of 20 mU m^{-2} min^{-1} for 120 minutes while clamping serum glucose at 5 mmol/L. From 120 to 240 minutes, we infused insulin at a rate of 40 mU m^{-2} min^{-1} while clamping

Table 1
Baseline characteristics of the subjects (n = 9)

	Mean (SE)	Median	Range	95% CI
Age at enrollment (y)	33.1 (1.5)	32	26-39	29.6-36.6
Duration of diabetes (y)	7.1 (1.6)	6	1-14	3.3-10.9
Prepregnancy hemoglobin A_{1c} (%)	7.5 (0.7)	6.7	5.8-10.9	6.0-9.0
Prepregnancy BMI (kg/m^2)	33.6 (1.9)	33.8	23.6-42.5	29.1-38.0
BMI 2nd trimester (kg/m^2)	35.8 (1.8)	36.9	28.2-46.4	31.7-40.0
BMI 3rd trimester (kg/m^2)	38.8 (2.1)	38.7	30.6-48.1	35.0-44.8
Gestational age at delivery (wk)	37.5 (0.5)	38.0	35.0-40.1	36.4-38.7
Peripartum hemoglobin A_{1c} (%)	6.0 (0.3)	6.2	4.7-7.5	5.4-6.7
Birth weight (g)	3384 (247)	3345	2420-5035	2863-4092
Birth weight (*z* score)	0.49 (0.59)	0.31	−2.0 to +3.8	−0.88 to +1.86

Table 2
Glucose and hormonal concentrations in the fasting state and during 3 consecutive clamps in pregnant subjects with diabetes (second trimester of pregnancy)

	Fasting	Clamp 1: 0-120 min, insulin infusion: 20 mU m^{-2} min^{-1}, glycemia clamped at 5 mmol/L	Clamp 2: 120-240 min, insulin infusion: 40 mU m^{-2} min^{-1}, glycemia clamped at 5 mmol/L	Clamp 3: 240-360 min, insulin infusion: 40 mU m^{-2} min^{-1}, glycemia clamped at 10 mmol/L
Glucose (mmol/L)	5.0 (0.33) [4.3-5.8]	5.03 (0.04) [4.9-5.1]	4.85 (0.05) [4.7-5.0]	10.0 (0.01) [9.9-10.0]
Insulin (pmol/L)	269 (46) [163-376]	438 (59) [318-558]	570 (53) [450-692]	728 (87) [526-929]
AG (pg/mL)	25.1 (5.2) [13.3-37.0]	27.4 (4.8) [16.4-38.4]	31.0 (6.9) [15.2-46.8]	26.0 (5.3) [13.8-38.1]
DG (pg/mL)	249 (35) [168-330]	190 (22) [138-241]	192 (23) [140-244]	168 (22) [116-219]
Leptin (ng/mL)	71 (7) [56-87]	66 (6) [52-80]	65 (6) [51-78]	64 (5) [52-76]
Adiponectin (μg/mL)	6.6 (1.5) [3.1-10.0]	6.1 (1.4) [2.9-9.2]	5.8 (1.2) [3.1-8.6]	5.8 (1.4) [2.7-9.0]

The *M*/*I* ratio measured at the end of the euglycemic-normoinsulinemic clamp (20 mU insulin m^{-2} min^{-1}) was 0.0036 (SE, 0.0010) mg kg^{-1} min^{-1} of glucose infused per picomole per liter of plasma insulin [95% CI, 0.0015-0.0057]. The *M*/*I* ratio was 0.0056 (SE, 0.0014) [95% CI, 0.0025-0.0087] for the euglycemic-hyperinsulinemic clamp (40 mU insulin m^{-2} min^{-1}). Desacyl ghrelin concentrations decreased with increasing glucose and insulin concentrations ($P = .004$, n = 9). There was a 33% decrease in DG between the fasting state and the hyperglycemic-hyperinsulinemic clamp. Acyl ghrelin concentrations were not significantly different between the fasting state and the clamps. The correlation between fasting leptin and adiponectin did not reach statistical significance ($r_s^2 = 0.34$, $P = .09$). n = 9, mean (SE) [95% CI].
Conversion factors:
Glucose: to convert millimoles per liter to milligrams per deciliter, multiply by 18.
Insulin: to convert picomoles per liter to microunits per milliliter, divide by 6.
Acyl ghrelin: to convert picograms per milliliter to picomoles per liter, divide by 3.371.
Desacyl ghrelin: to convert picograms per milliliter to picomoles per liter, divide by 3.189.

serum glucose at 5 mmol/L. Finally, between 240 and 360 minutes, we infused insulin at a rate of 40 mU m^{-2} min^{-1} while clamping glucose at 10 mmol/L (Tables 2 and 3). Glucose was measured immediately at the bedside at 5-minute intervals, with adjustment of the infusion rate to maintain plasma glucose at the appropriate level. Circulating insulin levels were measured every 20 minutes. At 100, 220, and 340 minutes, blood was collected for the determination of AG, DG, leptin, and adiponectin.

2.2. Sample collection and assays

Plasma glucose was measured using a YSI glucose analyzer (YSI Life Sciences, Yellow Springs, OH). All other blood samples (collected in tubes containing 1.25 mg sodium EDTA per milliliter of whole blood) were kept on ice and centrifuged within 20 minutes at 1500 *g* and 4°C for 15 minutes. Plasma ghrelin was detected using a 2-site sandwich assay that specifically detects full-length forms

Table 3
Glucose and hormonal concentrations in the fasting state and during 3 consecutive clamps in pregnant subjects with diabetes (third trimester of pregnancy)

	Fasting	Clamp 1: 0-120 min, insulin infusion: 20 mU m^{-2} min^{-1}, glycemia clamped at 5 mmol/L	Clamp 2:120-240 min, insulin infusion: 40 mU m^{-2} min^{-1}, glycemia clamped at 5 mmol/L	Clamp 3: 240-360 min, insulin infusion: 40 mU m^{-2} min^{-1}, glycemia clamped at 10 mmol/L
Glucose (mmol/L)	4.80 (0.47) [3.7-5.9]	5.26 (0.20) [4.8-5.7]	5.04 (0.13) [4.7-5.3]	10.08 (0.05) [9.9-10.2]
Insulin (pmol/L)	392 (97) [163-622]	612 (115) [340-883]	700 (117) [422-977]	987 (186) [548-1427]
AG (pg/mL)	32.4 (12.4) [3.2-61.6]	31.8 (13.6) [0-64]	35.7 (14.1) [2.4-69.0]	32.7 (12.3) [3.7-61.8]
DG (pg/mL)	178 (33) [99-257]	134 (21) [84-183]	134 (17) [94-174]	123 (18) [80-166]
Leptin (ng/mL)	86 (10) [61-110]	78 (11) [52-105]	74 (11) [49-100]	69 (10) [45-93]
Adiponectin (μg/mL)	6.0 (1.2) [3.1-8.8]	5.6 (1.1) [3.0-8.1]	5.4 (1.1) [2.7-8.0]	5.4 (1.2) [2.6-8.1]

The *M*/*I* ratio measured at the end of the euglycemic-normoinsulinemic clamp (20 mU insulin m^{-2} min^{-1}) was 0.0027 (SE, 0.0011) [95% CI, 0.0002-0.0053] during the third trimester. The *M*/*I* ratio was 0.0045 (SE, 0.0012) [95% CI, 0.0018-0.0072] for the euglycemic-hyperinsulinemic clamp (40 mU insulin m^{-2} min^{-1}). Baseline DG concentrations decreased from the second to the third trimester of pregnancy ($P = .04$). Desacyl ghrelin concentrations also decreased with increasing glucose and insulin concentrations ($P = .09$, n = 7), although the decrease only reached statistical significance in the second trimester. Overall, there was a 27% decrease in DG between the fasting state and the hyperglycemic-hyperinsulinemic clamp. Acyl ghrelin concentrations were not significantly different in the fasting state or during the clamps. Fasting leptin concentrations increased significantly between the second and third trimesters of pregnancy ($P = .018$), whereas adiponectin concentrations remained similar. There was a positive correlation between fasting leptin and adiponectin ($r_s^2 = 0.69$, $P = .01$), but leptin and adiponectin were not affected by the changes in glucose and insulin concentrations. There was also a correlation between birthweight *z* score and the percentage of fasting AG that was acylated during the third trimester ($r_s^2 = 0.73$, $P = .007$).
n = 8 for fasting values, 7 for clamped values, mean (SE) [95% CI].
Conversion factors:
Glucose: to convert millimoles per liter to milligrams per deciliter, multiply by 18.
Insulin: to convert picomoles per liter to microunits per milliliter, divide by 6.
Acyl ghrelin: to convert picograms per milliliter to picomoles per liter, divide by 3.371.
Desacyl ghrelin: to convert picograms per milliliter to picomoles per liter, divide by 3.189.

of both AG and DG. The sensitivity and intraassay coefficient of variation (CV) for AG and DG have been published [5]. Because of AG instability, special precautions were taken during sample collection to protect against plasma esterases [21]. After centrifugation, 10 μL of phenylmethanesulfonyl fluoride, 10 mg/mL solution in ethanol, and 50 μL of 1 mol/L HCl were added per milliliter plasma. Insulin was measured in duplicate by radioimmunoassay (HI-14K; Linco, St Charles, MO). The *M*/*I* ratio was defined as the ratio of the steady-state glucose disposal rate (*M*, in milligrams per kilogram per minute) over the steady-state concentration of insulin (*I*, in picomoles per liter) and was calculated at the end of the euglycemic (between 100 and 120 minutes) and hyperinsulinemic clamps (between 220 and 240 minutes). Steady state of insulin level or glucose infusion rate was calculated as the average of the values obtained during the last 20 minutes of each 120-minute interval. Leptin and total adiponectin concentrations were measured using commercially available kits. According to the manufacturer's data (Alpco Diagnostics, Salem, NH), the interassay CV is 6.8% and the intraassay CV is 5.5% for the leptin enzyme-linked immunosorbent assay kit. For the adiponectin enzyme-linked immunosorbent assay kit, the interassay CV is 5.0% and the intraassay CV is 5.4%.

2.3. Statistical analysis

Data are expressed as mean ± SEM. We used nonparametric testing for related samples to examine the effect of time (fasting and each of the 3 clamps) in each trimester (Friedman test) and the effect of pregnancy trimester (second vs third trimester, Wilcoxon). A P value < .05 was considered significant. A sample size of 9 subjects provided 80% power to detect a shift in the mean value by 0.75 standard deviation units at an α level of .05, based on samples paired within subjects. We used Spearman rank correlation coefficient (r_s) to examine the relationships among leptin and adiponectin, as well as between birthweight SDS and ghrelin. Data were analyzed with SPSS version 17.0 (SPSS, Chicago, IL; 2008).

3. Results

Characteristics of the subjects are presented in Table 1. Tables 2 (second trimester) and 3 (third trimester) show fasting glucose and hormonal concentrations alongside those during the steady state period of each of the 3 successive clamps. Two subjects were excluded from analysis of the second clamp study, one because she failed to return and one because of technical difficulties with the infusion rate that failed to cause an increase in insulin concentrations.

3.1. Glucose and insulin concentrations

Glucose concentrations were very close to target values during all 6 clamps, and insulin progressively increased during the 3 consecutive phases of the clamps (P < .001). Overall, insulin concentrations increased from the second to the third trimester of pregnancy (P = .011). Insulin sensitivity was not significantly different between the second and third trimesters of pregnancy. The *M*/*I* ratio measured at the end of the euglycemic-normoinsulinemic clamp (20 mU insulin m^{-2} min^{-1}) was 0.0036 (0.0010) mg kg^{-1} min^{-1} of glucose infused per picomole per liter of plasma insulin during the second trimester [95% confidence interval {CI}, 0.0015-0.0057] and 0.0027 (0.0011) [95% CI, 0.0002-0.00530] during the third trimester. These values were 0.0056 (0.0014) [95% CI, 0.0025-0.0087] and 0.0045 (0.0012) [95% CI, 0.0018-0.0072] for the euglycemic-hyperinsulinemic clamp (40 mU insulin m^{-2} min^{-1}).

3.2. Full-length AG and DG concentrations

Baseline DG concentrations decreased from the second to the third trimester of pregnancy (P = .04). Desacyl ghrelin concentrations also decreased with increasing glucose and insulin concentrations (P = .004 during the second trimester, n = 9; P = .09 during the third trimester, n = 7), although the decrease only reached statistical significance in the second trimester. Overall, there was a 27% (third trimester) to 33% (second trimester) decrease in DG between the fasting state and the hyperglycemic-hyperinsulinemic clamp, whereas glucose concentrations doubled (from 5 to 10 mmol/L) and insulin concentrations almost tripled (Tables 1 and 2). Acyl ghrelin concentrations were relatively low, were similar during the second and third trimesters of pregnancy, and were not significantly different in the fasting state or during the clamps. Individual subject responses for AG and DG are presented in Fig. 1.

3.3. Leptin and adiponectin

Fasting leptin concentrations increased significantly between the second and third trimesters of pregnancy (P = .018), whereas adiponectin concentrations remained similar. Leptin and adiponectin were not affected by the changes in glucose and insulin concentrations.

3.4. Correlations

There was a positive correlation between fasting leptin and adiponectin during the second (r_s^2 = 0.34, P = .09) and third (r_s^2 = 0.69, P = .01) trimesters that only reached significance during the third trimester. There was also a correlation between birthweight *z* score and the percentage of total ghrelin that was acylated during the third trimester of pregnancy (r_s^2 = 0.73, P = .007). There were no significant correlations between BMI and hormonal parameters before or during pregnancy.

4. Discussion

Using a ghrelin assay that recognizes both full-length AG and DG concentrations, we clarify the role of insulin and

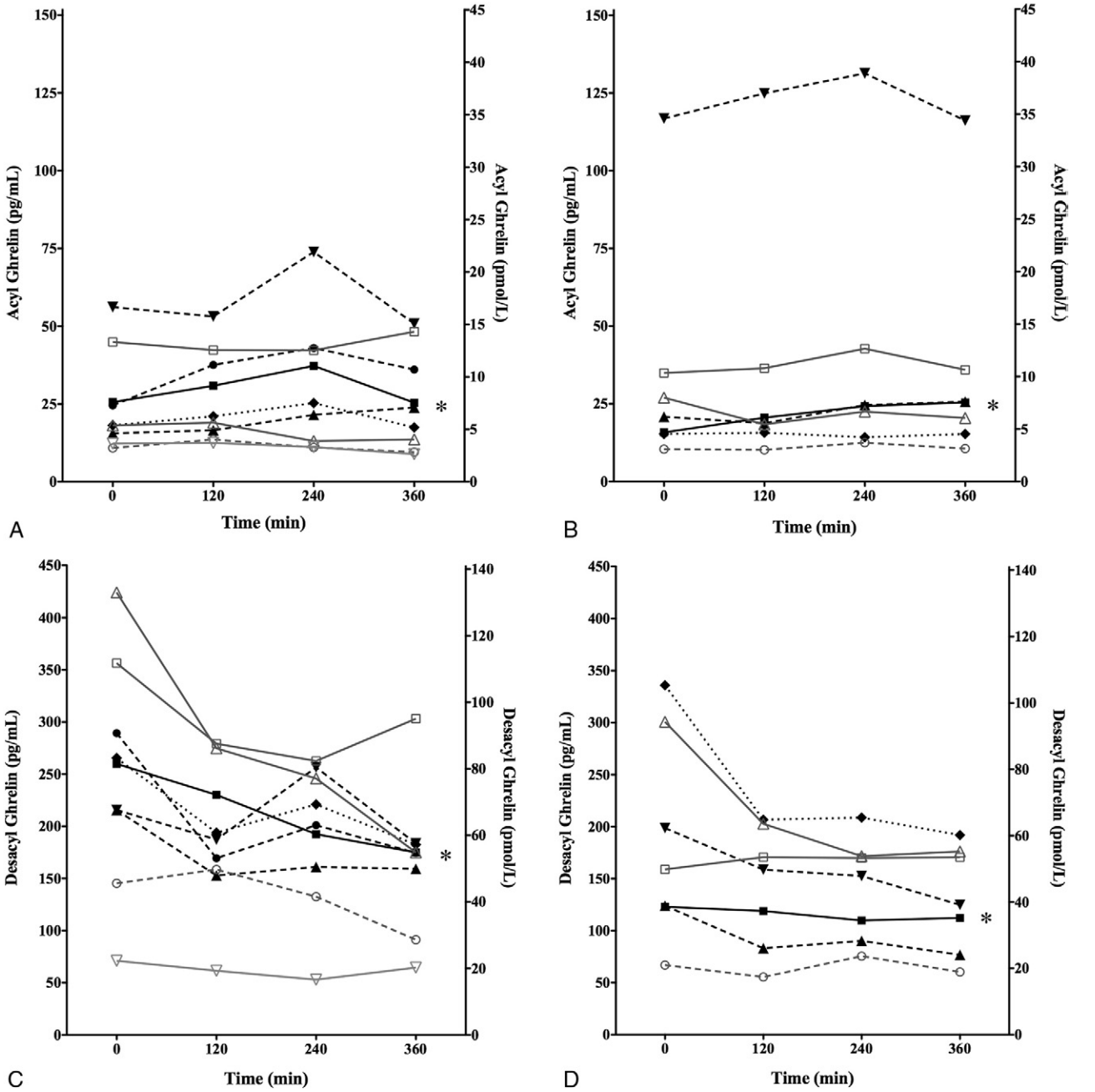

Fig. 1. Individual responses of AG (A and B) and DG (C and D) during the second (A and C, n = 9) and third (B and D, n = 7) trimesters of pregnancy in subjects with diabetes. The patient with mature-onset diabetes of the young is indicated with filled squares and asterisks, and was not an outlier. Baseline DG concentrations decreased from the second to the third trimester of pregnancy ($P = .04$). Desacyl ghrelin concentrations decreased with increasing glucose and insulin concentrations ($P = .004$ during the second trimester, n = 9; $P = .09$ during the third trimester, n = 7). There was a 27% (third trimester) to 33% (second trimester) decrease in DG between the fasting state and the hyperglycemic-hyperinsulinemic clamp. Acyl ghrelin concentrations were relatively low, were similar during the second and third trimesters of pregnancy, and were not significantly different in the fasting state or during the clamps. Conversion: AG: to convert picograms per milliliter to picomoles per liter, divide by 3.371; DG: to convert picograms per milliliter to picomoles per liter, divide by 3.189.

glucose on ghrelin concentrations in pregnant women affected with diabetes. We demonstrate that glucose and insulin cause a 27% to 33% decrease in circulating DG concentrations, without affecting AG concentrations. Desacyl ghrelin has an emerging role in energy balance regulation, although whether its primary effect is in the control of caloric intake [9], nutrient partitioning [11], glucose homeostasis [22], or energy expenditure remains to be determined.

Ghrelin is also emerging as a potentially important player during pregnancy. Animal studies have shown that AG administration to the pregnant dam caused an increase in birth weight, suggesting that maternal ghrelin could play an important role in perinatal growth. The increase in birth

weight was thought to be due to transplacental transfer of AG, was independent of maternal food intake, and was not observed when DG was administered to the dam [23]. Whether this is also true in humans is unknown, but our finding of a positive correlation between the percentage AG and birth weight SDS is consistent with this hypothesis. Interestingly, we have also observed a positive correlation between birth weight and percentage AG during the third trimester of pregnancy in another study investigating pregnant subjects with and without gestational diabetes ($r = 0.43$, $P = .034$, unpublished data) [5].

The low absolute concentrations of AG in the present study are similar to our data in pregnant women with and without gestational diabetes [5]. To better study the relationship between glucose homeostasis and ghrelin, we investigated a population characterized by impaired insulin secretion and sensitivity due to the additive effect of pregnancy and diabetes. Our design resulted in a progressive increase in insulin and glucose from the fasting state to the hyperglycemic-hyperinsulinemic clamp. However, despite a 2-fold increase in glucose concentrations and a 2- to 3-fold increase in insulin concentrations, AG concentrations remained unchanged throughout the study. In contrast, we observed a 27% to 33% decrease in DG concentrations as insulin and glucose concentrations progressively increased. Visual analysis of Fig. 1 (and of Tables 2 and 3) shows that the greatest decrease in DG occurs between fasting and the first clamp, and is associated with stable glucose concentrations in the face of a 60% increase in insulin concentrations, suggesting that insulin partly modulates these changes.

Several authors have used the glucose clamp to investigate the role of glucose and insulin on ghrelin concentrations, but this is the first such study performed in pregnant women. In addition, the hormone assays used in most published studies did not differentiate between AG and DG. Flanagan et al [24] observed an 18% decrease in total (AG + DG) ghrelin concentrations that was mostly the result of increased insulin concentrations and was independent of glycemia. Similar results were observed in women with and without polycystic ovarian syndrome [25] and in subjects with uncomplicated obesity [26] during a euglycemic-hyperinsulinemic clamp. A decrease in circulating total ghrelin levels was also seen by Anderwald et al [27] in subjects with type 2 diabetes mellitus and was found to be smaller than that in subjects without type 2 diabetes mellitus. In a study measuring both AG and DG concentrations (using a single-antibody method), St-Pierre et al [28] observed a decrease in DG concentrations in insulin-sensitive and insulin-resistant, nondiabetic obese subjects during a euglycemic-hyperinsulinemic clamp. Acyl ghrelin concentrations were found to be decreased only in insulin-sensitive obese subjects. Taken together, these data suggest that insulin causes a decrease in ghrelin concentrations that may be blunted by insulin resistance.

Our results also show that insulin inhibits DG during pregnancy. However, in the absence of a control group of nondiabetic pregnant women, we cannot determine whether insulin resistance reduces this response. Interestingly, AG was unaffected by changes in insulin and glucose concentrations during the clamps. This may reflect the presence of obesity and insulin resistance in our patients, 2 situations that have recently been reported to be associated with increased AG concentrations in nonpregnant subjects [11,28]. However, this could also be explained by the specific effect of pregnancy on AG concentrations. We have recently shown that circulating AG concentrations were lower in pregnant compared with nonpregnant subjects [5], a finding that may be due to decreased acylation of ghrelin in the stomach, at least in rodents (Chanoine, unpublished results). Finally, because AG concentrations are low during pregnancy and close to the limit of detection of our assay, we cannot rule out a small, unrecognized decrease in AG.

As expected during pregnancy [29], leptin levels increased significantly between the second and third trimesters. Fasting leptin concentrations were markedly higher in our study (mean [SD], 86 [29] ng/mL) than those reported by Butte et al [30] in healthy nondiabetic women (30 [17] ng/mL) during the third trimester of pregnancy. This likely reflected the greater BMI (and presumably fat mass) of our subjects (38.7 [5.9] vs 28.1 [3.8] kg/m^2). Interestingly, leptin concentrations were not affected by increasing insulin and glucose concentrations. In vitro data show that insulin stimulates leptin secretion by the adipocyte [31], but neither insulin nor glucose acutely stimulates leptin secretion by placenta explants [32]. In vivo, clamp studies performed in nonpregnant subjects have generally reported a stimulatory effect of insulin on circulating leptin [33], consistent with the in vitro effect mentioned above. However, this effect of insulin on leptin secretion is abolished by insulin resistance [33,34]. Taken together, the lack of a significant effect of insulin on leptin concentrations in our subjects likely reflects synergistic insulin resistance secondary to pregnancy as well as to diabetes.

Our small sample size reflects the difficulty of recruiting pregnant women for these studies and of performing clamp studies at 2 occasions during pregnancy. As a consequence, we did not have the power to detect small differences in ghrelin concentrations between the various phases of the clamp or to differentiate between the respective effects of insulin and glucose. In addition, several AG measurements were close to the detection limit of the assay, which would further limit our ability to demonstrate insulin-induced suppression of AG. We did not study a control group of nondiabetic pregnant women, as recruitment of healthy pregnant women for these procedures was deemed unethical. Nonetheless, our study presents several strengths. First, we achieved 3 different combinations of glucose and insulin steady-state concentrations, providing a wide range of glucose and insulin concentrations. Second, the subjects were investigated in 2 different trimesters of pregnancy.

In conclusion, we show that acute increases in insulin and glucose in pregnant subjects with diabetes are associated

with a 30% decrease in DG concentrations, without changes in their already suppressed AG. Emerging data show that DG affects energy expenditure. We speculate that changes in DG concentrations may contribute to impaired energy balance in pregnant women with poor control of diabetes.

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Declaration of competing interests: nothing to declare.

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