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Postprandial Suppression of Glucagon Secretion Depends on Intact Pulsatile Insulin Secretion

Further Evidence for the Intraislet Insulin Hypothesis

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Type 2 diabetes is characterized by an ~60% loss of β-cell mass, a marked defect in postprandial insulin secretion, and a failure to suppress postprandial glucagon concentrations. It is possible that postprandial hyperglucagonemia in type 2 diabetes is due to impaired postprandial insulin secretion. To address this, we studied eight adult Goettingen minipigs before and after an ~60% reduction in β-cell mass induced by alloxan. Pigs were studied fasting and after ingestion of a mixed meal. Insulin and glucagon secretion were determined by deconvolution of blood hormone concentrations measured at 1-min intervals. The relationship between insulin and glucagon release was analyzed using cross-correlation and forward versus reverse cross–approximate entropy. We report that glucagon and insulin were secreted in ~4-min pulses. Prealloxan, postprandial insulin secretion drove an ~20% suppression of glucagon concentrations ($P < 0.01$), through inhibition of glucagon pulse mass. The alloxan-induced ~60% deficit in β-cell mass lead to an ~70% deficit in postprandial insulin secretion and loss of the postprandial insulin-driven suppression of glucagon secretion. We conclude that postprandial hyperglucagonemia in type 2 diabetes is likely due to loss of intraislet postprandial suppression of glucagon secretion by insulin. *Diabetes* 55:1051–1056, 2006

Hyperglucagonemia is a characteristic of both type 1 and type 2 diabetes (1–3). In the overnight-fasted state, plasma glucagon concentrations in patients with diabetes are often comparable to those of nondiabetic individuals but are inappropriately high in the setting of hyperglycemia (1). After glucose ingestion, there is impaired glucagon suppression in both type 1 and type 2 diabetes (4,5). Postprandial hyperglucagonemia is an important contributor to failed suppression of hepatic glucose release after meal ingestion (3,6). Several hypotheses have been expounded to account for hyperglucagonemia in people with diabetes. Type 1 and type 2 diabetes are both characterized by a deficit in β-cell mass (7–11) and impaired postprandial insulin secretion (6,12,13). This is consistent with the role of the deficient intraislet insulin on postprandial suppression of glucagon secretion (14–16).

To address this, we studied a porcine model of type 2 diabetes with an alloxan-induced ~60% deficit in β-cell mass (17). We measured, in 1-min intervals, circulating insulin and glucagon concentrations in the fasting state and then after ingestion of a mixed meal in the same pigs before and after the alloxan-induced reduction of β-cell mass. We analyzed these data to address the following questions: *1*) Is glucagon secreted in pulses, and if so, are these pulses modulated to regulate glucagon secretion? *2*) After ingestion of a mixed meal, does insulin secretion contribute to suppression of glucagon secretion? *3*) Does an ~60% decrease in β-cell mass lead to impaired insulin-mediated suppression of glucagon secretion?

RESEARCH DESIGN AND METHODS

Insulin secretion in relation to β-cell mass in these pigs was previously reported (17). The present studies focused on the relationship between insulin secretion and glucagon secretion in this porcine model.

Preparation of animals for study. All studies were approved by the animal research committee. A total of eight Gottingen minipigs (six males and two females) that were 1–3 years old and weighed 20–32 kg were studied. The animals were obtained from Ellegaards Gottingen Minipigs (Gottingen, Denmark), where they were kept in a barrier unit for the 1st year. The pigs were housed under controlled conditions, 12-h light/12-h dark, and fed twice daily a total of 450 g Minipigs Maintenance Diet (9.28 MJ/kg; SDS, Edinburgh, U.K.), 13.8% protein, 51.2% nitrogen-free extract (equivalent to carbohydrate content), 2.5% fat, 12.7% fiber, and 12% water and allowed free access to water. Body temperature, hematocrit, and weight were recorded once a week, and the animals were exercised on a daily basis. The pigs were studied at least 10 days after surgery when they were eating normally, had normal stools, were maintaining weight, and had normal hemoglobin and leukocyte count and normal renal and liver function. Before the study, all pigs were acclimatized to the laboratory and staff.

The left jugular vein and carotid artery were exposed by cut-down and were catheterized, and the free ends of the catheters were tunneled to the left dorsal interscapular area as described (17). The catheters were filled carefully to capacity with heparin (10,000 IU/ml) and color labeled for later identification. The catheters were protected by a vest. Ampicillin (20 mg/kg; SmithKline Beecham, Dublin, Ireland) was given intravenously pre- and postoperatively for infection prophylaxis. Buprenorphine, 0.01 mg/kg, was given by epidural infusion after induction of anesthesia as analgesic cover for 24 h. This was supplemented by a bolus of 4 mg/kg Zenecarp (Parke Davis, Eastleigh, U.K.) administered intravenously at the end of the surgical procedure. A mix of buprenorphine (0.01 mg/kg) and Zenecarp (2 mg/kg) was given intravenously twice daily postoperatively until the animal had recovered fully from the procedure. Body temperature was measured daily for 7 days postoperatively, and antibiotics were administered only when there were signs of postoperative infection. Patency of catheters was checked every day. The catheter

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ApEn, approximate entropy.

exteriorization site was cleaned regularly with hydrogen peroxide (4%), and Bactroban cream (SmithKline Beecham) was applied.

Reduction of β-cell mass with alloxan. β-Cell mass was reduced by intravenous injection of alloxan monohydrate (Sigma, Dorset, U.K.), as described (17). The alloxan was refrigerated in a dessicator until used; all alloxan doses were made up from the same batch. The alloxan was weighed out immediately before administration in a 0.1-N acetic acid solution (Sigma), pH 4.4. A dose of 80 mg/kg was diluted in the buffer to a volume of 1.2-ml stock solution/kg. Immediately after preparation, the solution was injected as a bolus through a 0.22-μm Millipore filter (Sigma) into the jugular vein over 1 min, immediately after which the animals were fed. All of the animals developed mild to moderate hyperglycemia (range 6–11 mmol/l) within the first 24 h after alloxan administration, but this was not treated with insulin until 48 h after alloxan administration. After 48 h, the animals were treated only with insulin (Velosulin; Novo Nordisk, Bagsvaerd, Denmark) if the fasting plasma glucose was >9 mmol/l (n = 1 animal) or if the animal had glucosuria (n = 1, the same animal). Plasma glucose was measured daily, and urinalysis was performed (Chemstrip, Boehringer Mannheim, U.K.) twice a week to check for glucose, ketones, pH, and protein. No pig developed ketonuria. The diabetic animal that received insulin had no insulin on the morning of the experiments.

α-Cell mass. To ensure that α-cell mass was not affected by alloxan treatment, α-cell mass was determined from pancreata that were retrieved at the end of the experimental series as described (17). The α-cell mass was calculated by the product of the α-cell fraction of pancreas cross-sectional area (determined by immunostaining using rabbit anti-glucagon antibody; 1:1,000 dilution; Immunostar, Hudson, WI) and the pancreatic weight. Because pigs served as their own controls (studied before and after alloxan administration), it was not possible to determine α-cell mass before alloxan administration. Therefore, three pigs that had not received alloxan were used as controls. Neither relative α-cell area (0.22 ± 0.07 vs. 0.14 ± 0.12%, respectively; P = 0.61) nor α-cell mass (0.12 ± 0.05 vs. 0.06 ± 0.05 g, respectively; P = 0.50) was reduced in the pigs that had received alloxan compared with the controls.

Experimental protocol pigs (n = 8) were trained to eat a mixed meal that contained glucose in sugar-free jelly (U.S. equivalent to Jell-O) mixed with Minipig Maintenance Diet. After a 16-h fast, pigs were placed in a restraining box and the sampling catheters were aspirated and flushed in preparation for sampling. A blood sample was obtained from the artery to determine the hematocrit. Saline, 0.9%, was infused via the jugular vein catheter at 30 ml/h throughout the study (from t = −40 to 90 min). At t = 0 min, a mixed meal consisting of 2.2 g/kg D-glucose in sugar-free jelly mixed with 50 g Minipig Maintenance Diet was provided. Blood samples (1.0 ml) were obtained from an arterial catheter at 1-min intervals for measurement of plasma insulin and glucagon. Arterial blood samples also were obtained at 5-min intervals for measurement of plasma glucose concentrations. One pig was studied in the fasting state only because the sampling catheter was clotting.

The samples for insulin and glucagon were drawn into chilled polyethylene tubes that contained EDTA, placed on ice, and centrifuged within 5 min of collection (2 min, 15,000 rpm). Plasma was separated into two tubes and frozen at −20°C for later analysis. The blood glucose samples were drawn into tubes that contained heparin and sodium fluoride, centrifuged, and measured immediately by the glucose oxidase technique (Beckman Glucose Analyzer 2) (Beckman, Fullerton, CA).

Assays. Insulin concentrations in plasma samples were measured in duplicate by an enzyme-linked immunosorbent assay (17) modified for porcine insulin. The assay is based on two monoclonal murine antibodies specific for intact human insulin. The operating range is from 5 to 2,000 pmol/l, recovery 85–120%, and the assay is linear up to 2,000 pmol/l. Glucagon samples were measured in duplicate by radioimmunoassay as described previously (17). Glucose was measured by the glucose oxidase technique using a Beckman Glucose Analyzer 2.

Data analysis

Deconvolution. Pulsatile secretion of glucagon was quantified by use of a deconvolution method developed by Veldhuis and Johnson (18). In brief, the 1-min plasma glucagon concentration time series were deconvolved by a multiparameter technique with the following assumptions. The arterial plasma glucagon concentration in each animal, as monitored at frequent intervals, results from five determinable and correlated parameters: *1*) a finite number of discrete glucagon secretory bursts occurring at specific times and having *2*) individual amplitudes (maximal rate of glucagon secretion within a burst), *3*) a common half-duration (duration of an algebraically Gaussian secretory profile) (19), superimposed on a *4*) basal time invariant glucagon secretory rate, and *5*) a monoexponential glucagon disappearance model in the systemic circulation. The last parameter consisted of an estimated half-life of 2.7 min as measured previously in the same animal model (19). Assuming the forgoing nominal glucagon disappearance values, we estimated the numbers, locations, amplitudes, and half-duration of glucagon secretory bursts, as well as a nonnegative basal glucagon secretory rate for each data set using nonlinear least square fitting of the multiparameter convolution for each glucagon time series. Secretory rates are expressed as mass units of glucagon (picograms) released per unit of distribution volume (milliliters) per unit of time (minutes). The mass of hormones secreted per burst (time integral of the calculated secretory burst) thus is computed as pictogram of glucagon released per liter of volume of distribution. The percentage of glucagon delivered into the circulation in discrete insulin bursts was calculated as described previously (20).

Cross-correlation. To assess the interrelationship between insulin and glucagon concentration time series, cross-correlation analysis was applied on a sample-by-sample basis. This procedure consists of linear correlations carried out repeatedly at various time lags between the paired values. Thus, each insulin concentration value is compared with a time-delayed measure (e.g., lag time minus 2 min) of a glucagon concentration value. At a zero time lag, simultaneous values are correlated.

Approximate entropy. Regularity of glucagon plasma concentration time series was assessed by the model-independent and scale-invariant statistic approximate entropy (ApEn). ApEn measures the logarithmic likelihood that runs of patterns that are close (within r) for m contiguous observations remain close (within the tolerance width r) on subsequent incremental comparisons. A precise mathematical definition has been prescribed by Pincus (21). ApEn is to be considered a family of parameters dependent on the choice of the input parameters m and r, and it is to be compared only when applied to time series of equal length. In this study, ApEn was calculated with r = 20% of the SD in the individual time series and m = 1, parameter choices that are standardly used in virtually all endocrinological applications of ApEn. A larger absolute value of ApEn indicates a higher degree on process randomness. ApEn is rather stable to noise that is withheld within the tolerance width r. There is a precedence for detrending the time series by first differencing before ApEn calculation.

Cross-ApEn statistics. To evaluate relative regularity of glucagon and insulin concentrations, we used the cross-approximate entropy statistic. Cross-ApEn is a bivariate analog of ApEn and quantifies joint pattern synchrony between two separate but linked time series after standardization (z score transformation of the data) (22,23). The interpretation of cross-ApEn as implemented here depends on whether pattern recurrence is assessed against the downstream or upstream sequence. By way of convention, we computed forward cross-ApEn using serial insulin concentrations as the template to assess pattern reproducibility in glucagon concentrations and calculated reverse cross-ApEn by using successive glucagon concentrations as the template to evaluate pattern recurrence in insulin concentrations. Note that, for physiological reasons, the foregoing schema of pairing relates the concentration of an input signal to the secretion rate of the output signal. The individual values for the forward cross-ApEn were compared with the respective reverse cross-ApEn values in each animal to identify the driving force in the interaction between the secretion of both hormones.

Statistical analysis. Data are presented as the mean and SE. Statistical analyses were carried out by paired one-way ANOVA using Statistica, version 6, (Statsoft, Tulsa, OK). A P value <0.05 was taken as evidence of statistical significance.

RESULTS

Glucose concentrations increased significantly after meal ingestion before alloxan treatment (Fig. 1; $P < 0.01$). This was accompanied by an approximately fivefold increase in insulin concentrations (Fig. 1; $P < 0.01$). As expected, alloxan treatment led to an increase in plasma glucose levels ($P = 0.067$ in the fasting state and $P < 0.05$ after meal ingestion).

Glucagon concentrations were suppressed after meal ingestion before alloxan treatment ($P < 0.01$ vs. fasting levels). In contrast, the postprandial suppression of glucagon concentrations was absent after alloxan treatment ($P = 0.64$). Moreover, both fasting and postprandial glucagon concentrations were significantly higher after the alloxan treatment ($P < 0.01$; Fig. 1).

Inspection of glucagon concentration time series clearly identified pulses occurring at ~4-min intervals both in the fasting state and after meal ingestion (Fig.2). The presence of pulsatile glucagon secretion was confirmed in all pigs

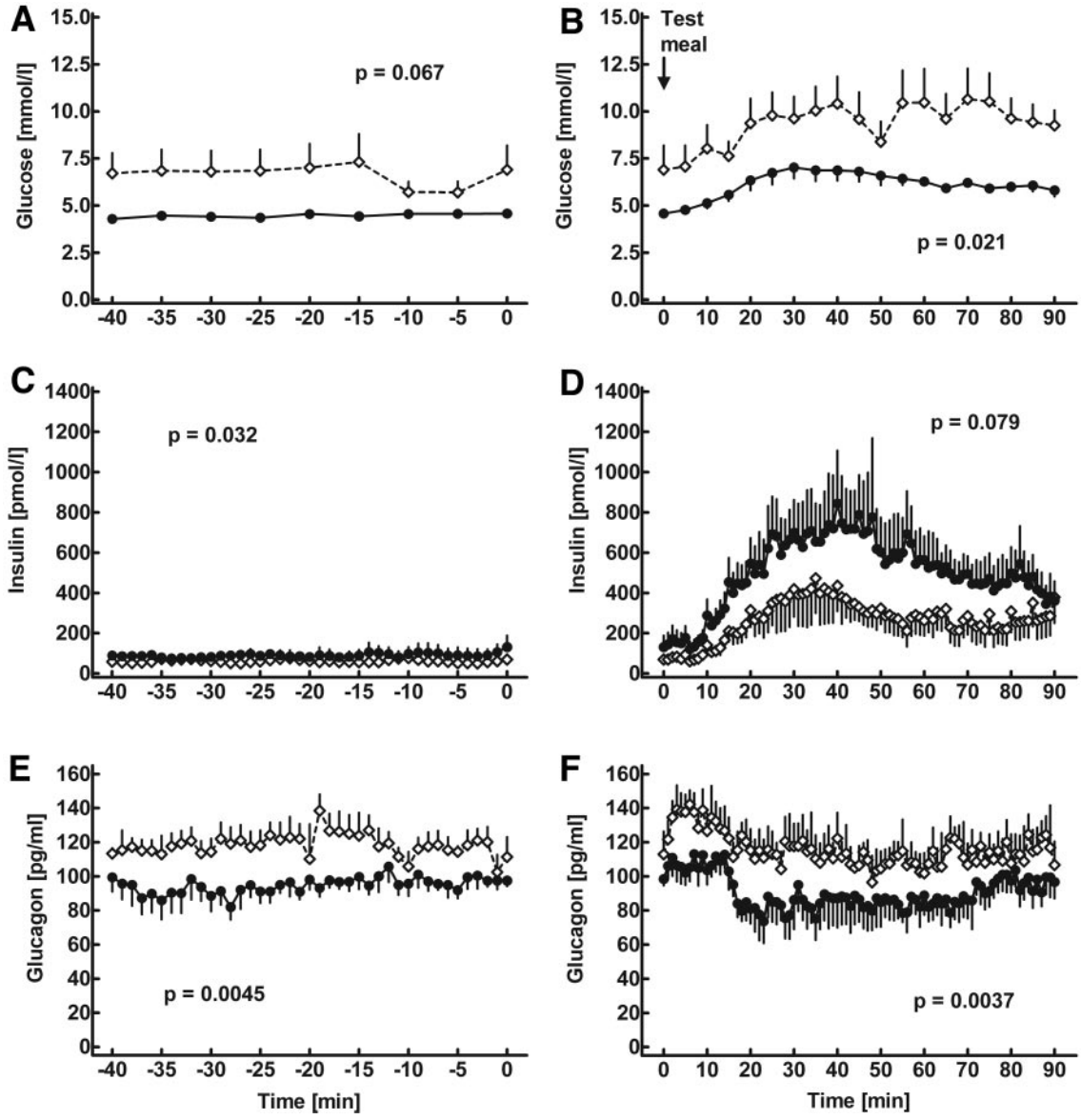

FIG. 1. Plasma concentrations of glucose (*A* and *B*), insulin (*C* and *D*), and glucagon (*E* and *F*) in eight pigs studied over 40 min in the fasting state (*left panels*) and over 90 min after meal ingestion (*right panels*). The same animals were studied before and after β-cell ablation by alloxan. Data are means ± SE. ◇, postalloxan; ●, prealloxan.

by deconvolution analysis both before and after alloxan treatment.

After the alloxan treatment, glucagon pulse mass increased from 30 ± 3 to 40 ± 4 pg/ml ($P < 0.01$), and pulse amplitude increased from 24 ± 3 to 32 ± 3 pg · ml^{-1} · min^{-1} ($P < 0.01$). In contrast, the glucagon pulse interval was not affected by alloxan treatment (3.8 ± 0.2 vs. 4.0 ± 0.1 min, respectively; $P = 0.17$).

The degree of randomness in the glucagon concentration time series, as measured by the ApEn value, was significantly higher after meal ingestion than during the fasting state both before (1.48 ± 0.09 vs. 1.25 ± 0.07, respectively; $P < 0.05$) and after the alloxan treatment (1.49 ± 0.07 vs. 1.27 ± 0,13, respectively; $P < 0.01$).

When glucagon and insulin concentration time series after meal ingestion were analyzed by cross-correlation analysis in the prealloxan pigs, we observed an inverse association between both hormones ($P < 0.05$; Figs. 3 and 4). Therefore, low glucagon concentrations were predicted by high insulin levels and vice versa.

To identify which hormone directed this relationship, we performed cross-ApEn analyses in a forward fashion by computing cross-ApEn using serial insulin concentrations as the template to assess pattern reproducibility in glucagon concentrations and in a reverse fashion using glucagon concentrations as the template to assess pattern reproducibility in insulin concentrations. Forward cross-ApEn was significantly higher than reverse cross-ApEn ($P < 0.01$), indicating that in healthy pigs, after meal ingestion, insulin drove glucagon concentrations.

In contrast, the postprandial association (cross-correlation) between insulin and glucagon concentrations was absent after alloxan-induced diabetes (NS; Fig. 4). Consistent with this, forward and reverse cross-ApEn analysis did not identify insulin as a driving force of glucagon levels after meal ingestion in the diabetic pigs ($P = 0.80$; Fig. 5).

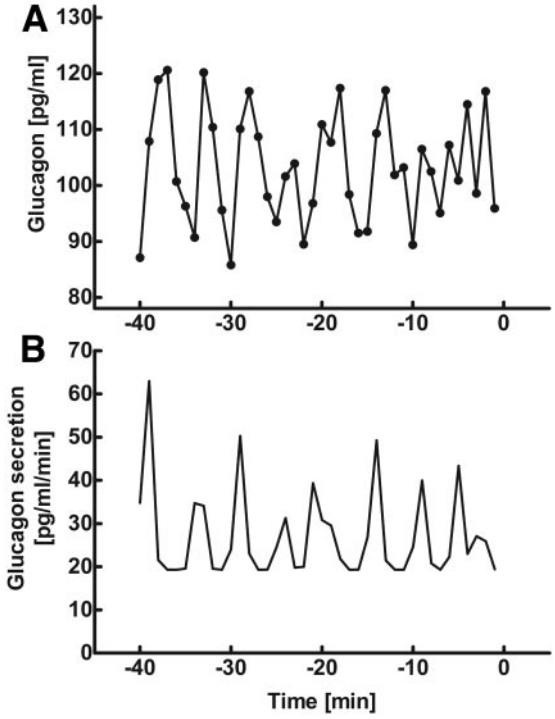

FIG. 2. Glucagon concentration time series (*A*) and corresponding glucagon secretion rates as assessed by deconvolution analysis (*B*) in a representative pig studied over 40 min in the fasting state before alloxan treatment.

These analyses therefore imply that in the postprandial state, pulsatile insulin secretion directly suppresses glucagon secretion, but this association is lost after an ~60% reduction in β-cell mass.

DISCUSSION

In the present studies, we sought to elucidate the mechanism of postprandial hyperglucagonemia in diabetes. We studied a porcine model of diabetes with an alloxan-induced ~60% deficit in β-cell mass and postprandial hyperglucagonemia (17). To investigate the mechanisms leading to this postprandial hyperglucagonemia, we first examined glucagon secretion before and after meal ingestion in the same pigs before alloxan.

Although it has been shown previously that circulating glucagon concentrations oscillate (24–26), here we deconvolved the glucagon concentrations to reveal that glucagon secretion is indeed pulsatile, and that, in common with insulin (27–29), glucagon secretion is regulated through modulation of pulse mass rather than pulse frequency. Thus, in healthy animals, glucagon secretion decreases after meal ingestion through the mechanism of suppression of glucagon pulse mass. While the latter is presumably mediated in large part through hyperglycemia, failure of glucagon suppression in people with diabetes has led to the hypothesis that the increment in postprandial intraislet insulin concentration also contributes to suppression of glucagon secretion (30–32).

The intraislet insulin hypothesis proposes that α-cell glucagon secretion is tonically inhibited by the release of insulin from the β-cell–enriched core of the islet (14,33,34). Indeed, because the blood flow in islets of Langerhans goes from the core to the mantle, the α-cells at the islet periphery are presumably exposed to very high levels of insulin (31). Moreover, insulin secretion from

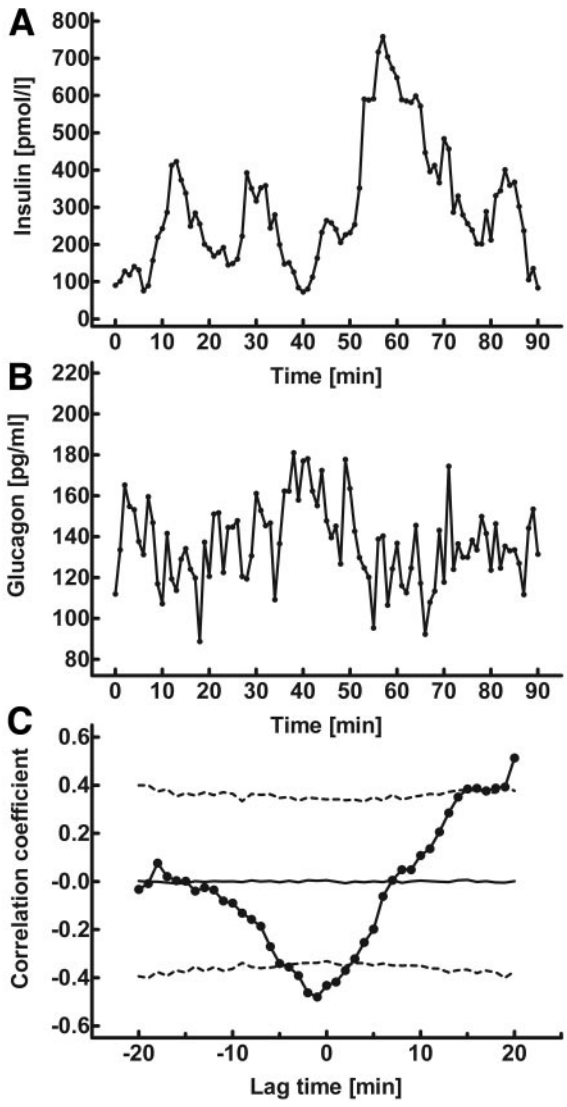

FIG. 3. Insulin (*A*) and glucagon (*B*) concentration time series in a representative pig studied over 90 min after meal ingestion before alloxan treatment illustrating the interdependency of both parameters. In *C*, the respective cross-correlogram (●) is depicted in relation to the upper and lower 95% CIs. This analysis demonstrates a significant cross-correlation between insulin and glucagon levels without detectable lag time.

β-cells arises from discrete secretory bursts occurring at ~4-min intervals (27,35). Regulation of insulin secretion is predominately accomplished by modulation of the mass of the insulin secretory bursts, increasing by approximately eightfold after glucose ingestion (36). The amplitude of the insulin oscillations present in the portal vein is ~1,000 pmol/l in the fasting state, increasing to ~4,000 pmol/l after glucose stimulation (37). Although it is not possible to directly measure the amplitude of the insulin concentration wavefront downstream of the islet β-cell core that affects α-cells, one can reasonably assume it is at least 100-fold greater than in the portal vein, since islet blood flow is <1% of total portal venous blood (38). By this estimate, in nondiabetic individuals, after meal ingestion, α-cells are exposed to insulin concentrations of ~400 nmol/l every 4 min. Also, the insulin receptor is expressed at high levels on pancreatic α-cells, and elimination of the insulin receptor abolishes glucagon responses to low glucose concentrations in pancreatic islets (39). In hu-

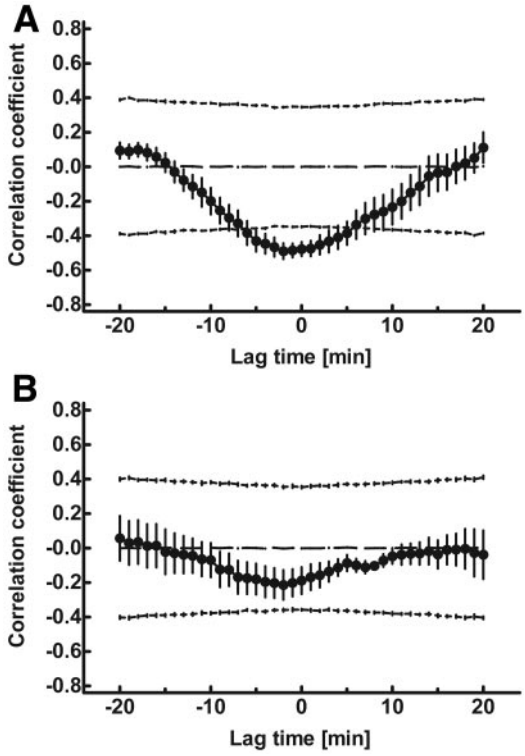

FIG. 4. Mean (± SE) cross-correlograms (●) between insulin and glucagon concentration time series in seven pigs studied over 90 min after a test meal, each before (*A*) and after (*B*) alloxan treatment. Data are presented in relation to the upper and lower 95% CIs (dotted lines). These analyses demonstrate a significant relationship between insulin and glucagon levels before alloxan treatment, whereas the time relationship between both hormones did not reach statistical significance after alloxan treatment.

mans, exogenous infusion of insulin reduces glucagon levels (40), and this effect is greater when insulin is administered in a pulsatile rather than a continuous manner (41).

In the present studies, we were able to support an important role of intraislet insulin concentrations on the responsiveness of α-cells to postprandial hyperglycemia through several lines of evidence. First, in healthy pigs after meal ingestion, we observed a significant cross-correlation between insulin and glucagon concentrations. Furthermore, using an unbiased statistical approach

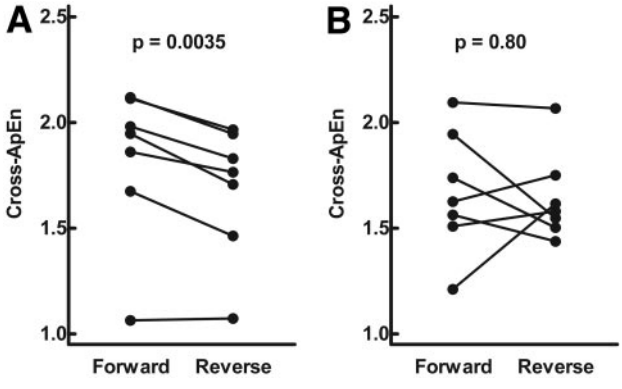

FIG. 5. Cross-ApEn statistics for the interaction of glucagon and insulin concentration time series in seven pigs studied in the postprandial period, each before (*A*) and after (*B*) alloxan treatment. Analyses were carried out by entering either glucagon (forward) or insulin (reverse) first.

(cross-ApEn), we were able to confirm that this relationship is due to the action of insulin on glucagon secretion. Moreover, when β-cell mass was decreased by ~60% in pigs, leading to a marked defect in postprandial insulin secretion, both the cross-correlation between insulin and glucagon and the direct action of insulin on glucagon secretion were no longer evident. Under these same circumstances, postprandial suppression of glucagon secretion was no longer present, despite an exaggerated postprandial glycemic excursion in the alloxan-treated pig.

The current studies, which support an important role of intraislet insulin-induced suppression of postprandial glucagon concentrations in health, are consistent with studies concluding that the decline in intraislet insulin is important to allow an appropriate glucagon response to hypoglycemia (14,16,32,34). Our findings concur with that model but in the reverse physiological circumstance of hyperglycemia-induced suppression of glucagon secretion.

Whereas a strong case is made here by the cross-ApEn analysis for insulin-driven suppression of glucagon secretion after meal ingestion, it is acknowledged that other secretory products of the β-cell, such as islet amyloid polypeptide and zinc, might have contributed to the suppression of glucagon secretion. Recent in vitro data have provided conflicting data as to whether zinc inhibits α-cell secretion via opening of ATP-sensitive K^+ channels (42,43).

In conclusion, the present data show that glucagon is secreted in distinct pulses in the fasting state and that the regulated suppression of glucagon secretion after meal ingestion is accomplished through suppression of glucagon pulse mass. We also report that, in health, suppression of glucagon secretion after meal ingestion is likely accomplished, at least in part, by increased insulin secretion. This action of insulin is lost in the alloxan model of type 2 diabetes, in which postprandial insulin secretion is impaired as a consequence of an ~60% decrease in β-cell mass. We conclude that postprandial hyperglucagonemia in diabetes is likely due at least in part to impaired insulin secretion, leading to loss of intraislet insulin-driven suppression of glucagon secretion.

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