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Objective: To examine whether lowering the plasma glucose concentration with empagliflozin (SGLT2 inhibitor) improves beta cell function in T2DM.
Research Design and Methods: 15 T2DM patients received empagliflozin (25 mg/day) for 2 weeks, and beta cell function was measured with 9-step hyperglycemic clamp (each step = +40 mg/dl) before and 48 hours and 14 days after empagliflozin.
Results: Empagliflozin caused 101±10 and 117±11 grams glucosuria on days 1 and 14 and produced 25±6 and 38±8 mg/dl reduction ($p<0.05$ for both) in fasting plasma glucose, respectively.
Empagliflozin increased the incremental area under the plasma C-peptide concentration curve by 48±12% and 61±10% during the stepped hyperglycemic clamp performed 48 hours and 14 days, respectively (both $p<0.01$), after the start of empagliflozin. Empagliflozin also caused an increase in the glucose infusion rate during the hyperglycemic clamp performed on days 3 and 14 compared to baseline by 15% and 16% (both $p<0.05$), respectively. Beta cell function, measured as the insulin secretion/insulin resistance (IS/IR) index, increased by 73±21% and 112±20% (both $p<0.01$) 48 hours and 14 days after the start of empagliflozin. Empagliflozin also enhanced beta cell glucose sensitivity during the hyperglycemic clamp by 42% and 54% after 48 hours and 14 days, respectively (both $p< 0.01$).
Conclusion: Lowering the plasma glucose concentration with empagliflozin in T2DM patients: (1) augments beta cell glucose sensitivity and (2) improves beta cell function (IS/IR index).

this study demonstrates that inhibition of renal glucose uptake with empagliflozin improves beta cell function measured with the gold standard technique, the hyperglycemic clamp.

INTRODUCTION

Progressive beta cell failure is the principal factor responsible for the development and progression of hyperglycemia in patients with type 2 diabetes mellitus (T2DM) (1-3). Multiple factors contribute to the development of beta cell failure including genetic and environmental factors (1-3). Chronic elevation of the plasma free fatty acid concentration markedly impairs insulin secretion in offspring of type 2 diabetic parents, i.e. lipotoxicity (4). Similarly, chronic elevation of the plasma glucose concentration has been shown to impair beta cell function. i.e. glucotoxicity (5-7). Conversely, reducing the plasma glucose concentration improves insulin secretion in both *in vivo* and *in vitro* studies in experimental animals (6,7). Lowering the plasma glucose concentration with insulin in T2DM individuals has been shown to improve beta cell function in humans (8-11). However, in addition to decreasing the plasma glucose concentration, insulin has multiple other metabolic effects including reduction of the plasma free fatty acid concentration which independently can improve beta cell function.

Sodium-glucose transporter-2 (SGLT2) inhibitors are a novel class of drugs which inhibit renal glucose absorption, thereby producing glucosuria and decreasing the plasma glucose concentration. We (12) previously have shown that lowering the plasma glucose concentration with an SGLT2 inhibitor reduces the plasma glucose concentration and improves insulin-mediated muscle glucose uptake (12). Because skeletal muscle does not express SGLT2, these results indicate that the improvement in insulin sensitivity caused by SGLT2 inhibition is secondary to amelioration of glucotoxicity. In preclinical studies in experimental diabetic animal models SGLT2 inhibitors have been shown to reduce/normalize the plasma glucose concentration and improve beta cell function (6). Recent studies using OGTT have demonstrted that, in T2DM patients, dapagliflozin and empagliflozin treatment for 2-4 weeks improves beta cell function (14,15).

The aim of the present study is to examine the effect of lowering the plasma glucose concentration with empagliflozin on beta cell function measured with the gold standard stepped hyperglycemic clamp technique.

MATERIALS AND METHODS

Subjects.

15 T2DM subjects with HbA1c = 7.8±0.2% and fasting plasma glucose (FPG) = 195±9 mg/dl participated in the study. Patient characteristics were: age = 55±2, 12 males/3 females, body mass index (BMI) = 31.1±2.1 kg/m^2, diabetes duration = 8.2±1.6 years, and eGFR = 107±7 ml/min ● 1.73m^2. Other than diabetes, subjects were in good general health as determined by medical history, physical exam, screening lab tests, and EKG. Body weight was stable (±3 pounds) in all subjects for at least 3 months prior to study and no subject participated in any excessively heavy exercise program. Diabetic subjects were treated with metformin (n = 11), metformin/sulfonylureas (n = 2), sulfonylurea (n=1), and diet (n=1). Other than these oral antidiabetic agents, subjects were not taking any medication known to affect glucose metabolism.

This was an open label study in which all diabetic subjects were treated with empagliflozin, 25 mg/day, and received a stepped hyperglycemic clamp before and 48 hours and 14 days after the start of empagliflozin.

Research Design:

All studies were performed on the Clinical Research Center of the Texas Diabetes Institute following an overnight fast. On days -5, -4, -2, and -1 before initiating empagliflozin treatment, 24-hour urine collections were performed for measurement of baseline urinary glucose excretion. On day -3, before initiating empagliflozin treatment, a baseline stepped-hyperglycemic clamp was performed following a 10-hour overnight fast. Subjects reported to the Clinical Research Center at 6AM and a catheter was placed into an antecubital vein. A low dose insulin infusion (0.1-0.2 mU/kg.min) was started to reduce the fasting plasma glucose concentration to ~100 mg/dl. The insulin infusion then was discontinued for 20 minutes, at which time the stepped hyperglycemic clamp (16) was performed. A second catheter was placed into a vein on the dorsum of the hand for blood withdrawal and the hand was placed in a thermo-regulated box heated to 60°C to obtain arterialized blood samples. At 7:30 AM (-90 minutes) subjects were asked to void, the urine was discarded and a volume of water equivalent to the voided volume of urine was consumed. At time zero (9AM) subjects voided and a stepped hyperglycemic clamp was performed. The plasma glucose concentration was acutely raised and maintained at 40 mg/dl above the fasting level (i.e. from ~100 to 140 mg/dl) for 40 minutes, and urine was

collected from 0-40 minutes for measurement of urinary glucose excretion. From 40-80, 80-120, 120-160, 160-200, 200-240, 240-280, 280-320, and 320-360 minutes the plasma glucose concentration was acutely raised and maintained at 180, 220, 260, 300, 340, 380, 420, and 460 mg/dl, respectively, with a variable infusion of 20% dextrose solution. Urine was collected during each 40 minute period and the subject consumed an amount of water equal to the voided urine volume to ensure spontaneous voiding. All urine samples were analyzed for glucose. Plasma C-peptide and glucose concentrations were measured at 2, 4, 6, 8, and 10 minutes and every 5-10 minutes thereafter until 360 minutes.

On the day after the stepped hyperglycemic clamp (day zero), subjects were started on empagliflozin, 25 mg/day, which they took in the morning for 14 days. On day 2 (i.e., 48 hours after the start of empagliflozin) and on day 14, the stepped hyperglycemic clamp was repeated as described above. The same insulin infusion rate (0.1-0.2 mU/kg•min) was used to reduce the fasting plasma glucose concentration to ~100 mg/dl during both repeat hyperglycemic clamp studies. 24 hour urine collections for measurement of glucose excretion were obtained during the 48 hours (i.e., days 0 and 1) after the start of empagliflozin and on days 12 and 13.

Analytical Techniques:

Plasma and urine glucose concentration was determined by glucose oxidation method (Analox, Analox Instruments, Middlebrough, UK). Plasma C-peptide and insulin concentrations (Linco Research, St Louis, MO) were determined by radioimmunoassay.

Calculations and Statistical Analyses:

Since the increment in plasma glucose concentration (+40 mg/dl every 40 minutes) was the same in all subjects, insulin secretion during each step of the hyperglycemic clamp was calculated as the incremental area under the plasma C-peptide concentration during that step. Tissue glucose uptake during each step was calculated as the glucose infusion rate during the last 20 minutes of each hyperglycemic step minus urinary glucose excretion during the same time period. Under the combined effects of hyperinsulinemia plus hyperglycemia, we previously have shown that endogenous glucose production is completely/near-completely suppressed (17). Insulin sensitivity index during the hyperglycemic clamp was calculated as the glucose infusion rate (minus urinary glucose excretion) at each step divided by the plasma insulin concentration. Beta cell function was calculated as the insulin secretion/insulin resistance (disposition) index: product of ΔC-Pep$_{(0\text{-}360\ min)}$ and insulin sensitivity index. Beta cell glucose sensitivity was calculated as the slope of the line relating C-peptide secretion and the plasma glucose concentration during each hyperglycemic clamp step.

Values are expressed as mean ± SEM. C-peptide secretion, beta cell function, glucose infusion rate and beta cell glucose sensitivity on day 2 (i.e., 48 hours after the start of empagliflozin) and on day 14 were compared to baseline with paired t-test. Statistical significance was set at $p<0.05$.

To determine independent factors related to the increase in insulin secretion (ΔC-pep$_{0\text{-}360}$), we created a multivariate linear regression model with ΔC-pep$_{0\text{-}360}$ as the dependent variable and age, BMI, diabetes duration, HbA1c, baseline FPG, decrement in FPG, and improvement in insulin sensitivity (glucose infusion rate minus urinary glucose excretion) as independent variables.

The study protocol was approved by the Institutional Review Board of University of Texas Health Science Center in San Antonio, Texas and all subjects gave written informed voluntary consent prior to participation.

RESULTS

Before the start of empagliflozin, urinary glucose excretion (mean of four determinations prior to empagliflozin) was 20±3 grams/24 hours, rose to 95±13 and 97±10 grams/day ($p<0.0001$ vs baseline) on days 0 and 1 post-empagliflozin and remained elevated (117±11grams/day) on day 13. The fasting plasma glucose concentration (mean of 4 determinations performed on days -5, -4, -2, -1 prior to the start of empagliflozin) was 195±9 mg/dl and decreased to 169±11 mg/dl (Δ = 25±6) and to 165±7 mg/dl (Δ=29±6) at 24 hours and 48 hours after starting empagliflozin (both $P<0.001$). The FPG decreased to 157±8 (Δ = 38±8) mg/dl on day 14 ($p= 0.0005$). The fasting plasma C-peptide concentration declined slightly, but not significantly, during empagliflozin treatment from 3.5±0.3 ng/ml at baseline to 3.2±0.3, 3.2±0.3, and 3.2±0.3 ng/ml at 24 hours, 48 hours, and 14 days after the start of empagliflozin.

Plasma Glucose and C-Peptide Concentration

The increment in plasma glucose concentration during each of the three hyperglycemic clamp studies was similar (Figure 1).

C-peptide Secretion

Figure 2 depicts the plasma C-peptide concentration during the hyperglycemic clamp performed at baseline and on day 2 (i.e., 48 hours after the start of empagliflozin) and on day 14. C-peptide concentration increased significantly on day 2 after initiation of empagliflozin therapy and the increase persisted on day 14. The incremental area under the plasma C-peptide concentration curve [$\Delta C\text{-}Pep_{(0\text{-}360)}$] during the baseline hyperglycemic clamp was 23±5 ng/ml•h and increased to 32 ±7 and 37±8 ng/ml•h on day 2 and on day 14 ($p=0.02$ and <0.005, respectively) (Figure 3). The incremental area under the plasma C-peptide concentration curve during the first 10 minutes of the hyperglycemic clamp (which represents first phase insulin secretion) was not altered by empagliflozin (0.61±0.36, 0.21±0.35 ($p=0.30$) and 1.2±0.44 ($p=0.10$) at baseline, day 2 and day 14 respectively).

Beta Cell Function

Tissue glucose uptake (measured as the mean glucose infusion rate (minus urinary glucose excretion) during the hyperglycemic clamp was 3.36±0.29 mg/kg.min and increased by 14% and 19% (to 3.84±0.44 and 4.01±0.43) on day 2 and on day 14, respectively ($p<0.05$) (Figure 3). However, when divided by the mean plasma insulin concentration during the hyperglycemic clamp, tissue glucose uptake was not statistically significant. Beta cell function, measured with the insulin secretion/insulin resistance (disposition) index, increased by 73±20% and 106±20% on days 2 and 14, respectively (from 88±23 at baseline to 155±46 and 187±58 on days 2 and 14, both $p <0.01$) (Figure 3).

Beta Cell Glucose Sensitivity

The slope of the line relating the area under the plasma C-peptide concentration curve and the plasma glucose concentration at each step of the hyperglycemic clamp represents beta cell glucose sensitivity. During the baseline hyperglycemic clamp, the slope was 0.024±0.005 ng/ml per mg/dl. Empagliflozin caused a 42% (to 0.034±0.007) and 54% (to 0.037±0.007) increase in beta cell glucose sensitivity on day 2 (i.e., 48 hours after the start of empagliflozin) and on day 14, respectively (both $p<0.05$) (Figure 4).

Relationship Between the Increase in Beta Cell Function and Decrease in FPG Concentration

The increment (Δ) in plasma C-Peptide concentration during the hyperglycemic clamp and the insulin secretion/insulin resistance (IS/IR) index strongly correlated with the decrease in FPG

concentration on day 2 and day 14 (r=0.60 and 0.61, respectively, P=0.01) (Figure 2B). No significant relationship was observed between the decrease in FPG concentration and the increase in glucose infusion rate during the hyperglycemic clamp. In a multivariate linear regression model, only the decrease in FPG was a significant predictor of the increase in $\Delta C\text{-pep}_{0\text{-}360}$ at 14 days with standardized beta = 0.862, p=0.002, with r^2=0.65

DISCUSSION

The present study assessed the acute (48 hours) and chronic (14 days) effect of SGLT2 inhibition on beta cell function in type 2 diabetic patients and provides two novel findings. First, it documents for the first time in man, using the gold standard hyperglycemic clamp, a marked increase in beta cell sensitivity to glucose following a reduction in the plasma glucose concentration. Second, it delineates the rapid (48 hours) time-related effect of reversal of glucotoxicity on beta cell function.

Progressive beta cell failure is the principal factor responsible for the development and progression of hyperglycemia (1,18-20). Thus, therapies which improve/halt beta cell failure should be effective in producing a durable reduction in HbA1c. Although several antidiabetic classes (e.g. sulfonylureas, glinides, incretins, thiazolidinediones) augment insulin secretion only the GLP-1 receptor agonists (21) and thiazolidinediones (22,23) improve beta cell function on a long term basis. DPP4 inhibitors improve insulin secretion, but their effect on the beta cell is weak [24] and, not surprisingly, they do not produce a durable reduction in HbA1c (25-27).

SGLT2 inhibitors lower the plasma glucose concentration via a mechanism which is independent of insulin secretion and insulin action. By inhibiting renal glucose reabsorption, they cause glucosuria, leading to a decline in fasting and postprandial plasma glucose concentration. Despite the lack of a direct effect on the beta cell, preclinical studies in animal models of diabetes have demonstrated improved beta cell function secondary to reduction of the plasma glucose concentration and amelioration of glucotoxicity (6). Using mathematical modeling, both canagliflozin (28) and empagliflozin (15) have been shown to improve beta cell function during the OGTT in T2DM patients. Ipragliflozin also has been shown to improve insulin secretion indices during the OGTT in T2DM patients (29), and we have shown an improvement in the insulin secretion/insulin resistance index during the OGTT in a small group of T2DM patients treated with dapagliflozin (14). No previous study has employed the hyperglycemic clamp to quantitate insulin secretion acutely (48 hours) and more chronically (14 day) following treatment with an SGLT2 inhibitor. In the present study, we employed the gold standard hyperglycemic clamp technique to quantitate beta cell function and demonstrated that a modest decrease in fasting plasma glucose concentration for as little as 48 hours after the initiation of empagliflozin therapy resulted in a robust increase in beta cell function. A 29 mg/dl decrement (48 hours after the start of empagliflozin) and 38 mg/dl decrement (14 days of the start of empagliflozin) in FPG resulted in a 43% and 74% increase, respectively, in C-peptide secretion during the hyperglycemic clamp; beta cell function (insulin secretion/insulin resistance index) increased by 73% and 106%, respectively. Remarkably, the improvement in C-peptide secretion observed with empagliflozin was evident 48 hours after starting the SGLT2 inhibitor, indicating that the beneficial effect of reversal of glucotoxicity on beta cell function is rapid in onset. Insulin secretion and insulin sensitivity are inversely related (30). We previously have shown that lowering the FPG concentration with an SGLT2 inhibitor in T2DM subjects improves insulin sensitivity. If anything, this would be expected to reduce C-peptide secretion. Thus, the increase in C-peptide secretion observed in the present study caused by empagliflozin represents a

primary effect on the beta cell, most likely secondary to reversal of glucotoxicity, and not a secondary effect due to change in insulin sensitivity. One other factor that could explain the improvement in beta cell function relates to the observation that glucose lowering improves the effect of incretins on insulin secretion (31). Although not examined in the present study, this possibility is worthy of exploration in future studies.

Although SGLT2 inhibitors do not exert a direct effect on the beta cell, we (13) and others (15) have demonstrated that this class of drugs alters fuels metabolism, causing a reduction in whole body glucose oxidation and a reciprocal increase in lipid oxidation. If the increase in lipid oxidation were to occur in the beta cell, this could lead to a decrease in beta cell lipid content and reversal of lipotoxicity (32). During the hyperglycemic clamp, empagliflozin caused a modest increase in glucose infusion rate, which was not significant when divided by plasma insulin concentration. We (13) and others (15) have shown that glucosuria produced by SGLT2 inhibition stimulates a compensatory increase in the rate hepatic glucose production. Because the total body rate of glucose appearance was not measured with labeled glucose in the present study, we could not quantitate the residual rate of hepatic glucose production during the hyperglycemic clamp. Therefore, it is not possible to reach any definitive conclusion about the effect of empagliflozin on insulin sensitivity in the present study.

In summary, lowering the plasma glucose concentration with empagliflozin causes a robust increase in beta cell function in T2DM patients. This effect of empagliflozin is rapid in onset and lasts for the entire treatment period (14 days).

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AUTHORS CONTRIBUTIONS

All authors contributed to performance of the study. Drs. Abdul-Ghani and DeFronzo wrote the initial draft of the manuscript which then was reviewed and revised by all of the authors.

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Figure 1. Plasma glucose concentrations during the three hyperglycemic clamp studies performed at baseline, on day 2 and on day 14 after the start of empagliflozin therapy.

Figure 2. [A] Plasma C-peptide response during the stepped hyperglycemic clamp performed at baseline and on day 3 and day 14 after the start of empagliflozin therapy. [B] Correlation between the decrease in the fasting plasma glucose concentration versus the log of the ratio of the plasma C-peptide response (AUC) after 14 days of empagliflozin to the plasma C-peptide response (AUC) at baseline. AUC = area under the curve.

Figure 3. [A] Incremental AUC for the plasma C-peptide response at baseline and on day 3 and day 14 after the start of empagliflozin treatment. [B] Glucose infusion rate (GIR) at baseline and on day 3 and day 14 after the start of empagliflozin. [C] The insulin secretion/insulin resistance (IS/IR) index at baseline and on day 3 and day 14 after the start of empagliflozin treatment. ($p < 0.05$ for both)

Figure 4. Slope of the line relating the increment in plasma C-peptide concentration and the increment in plasma glucose concentration during the stepped hyperglycemic clamp.

Figure 1

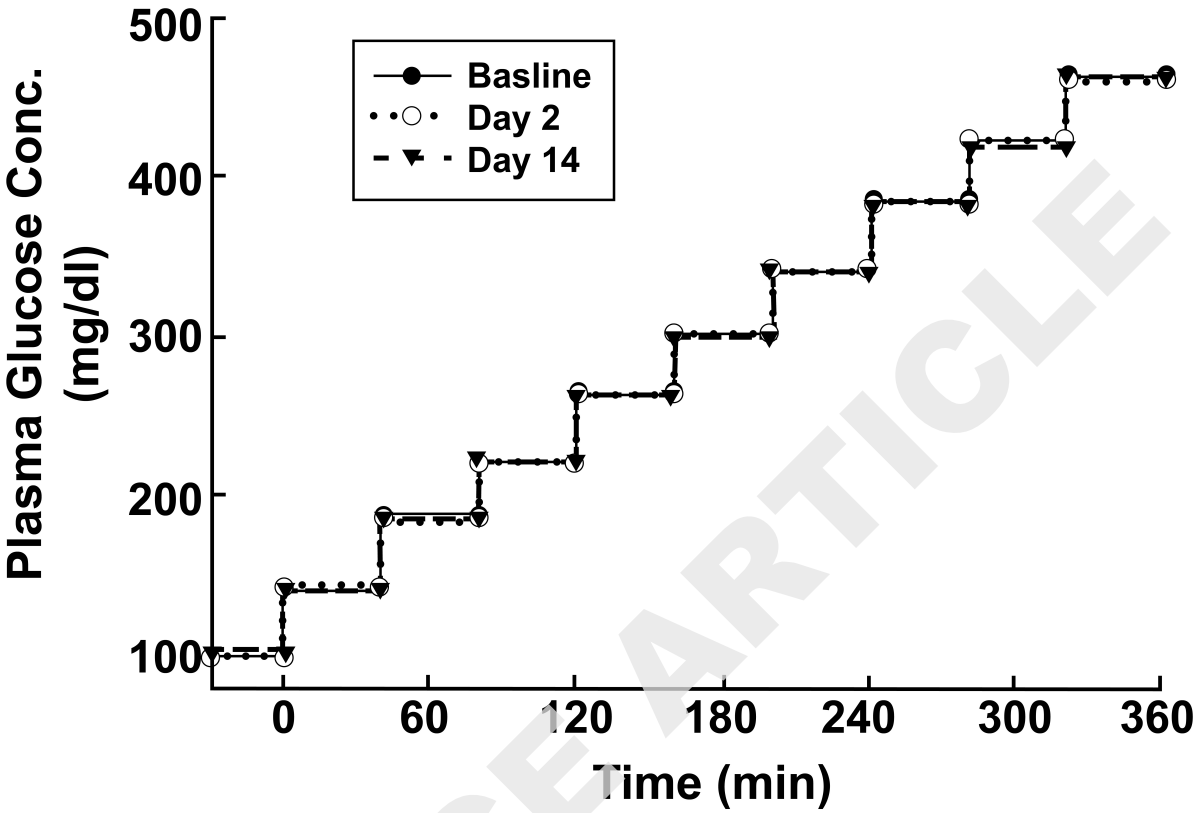

Figure 2A

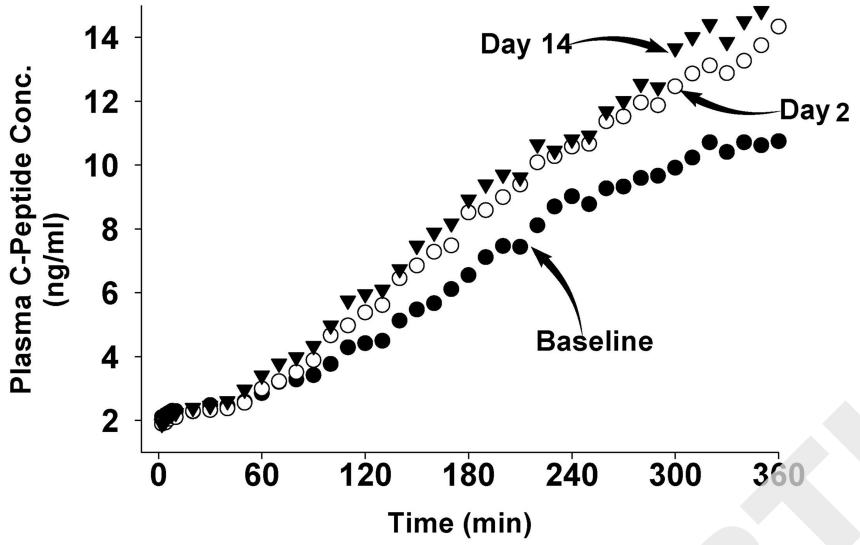

Figure 2B

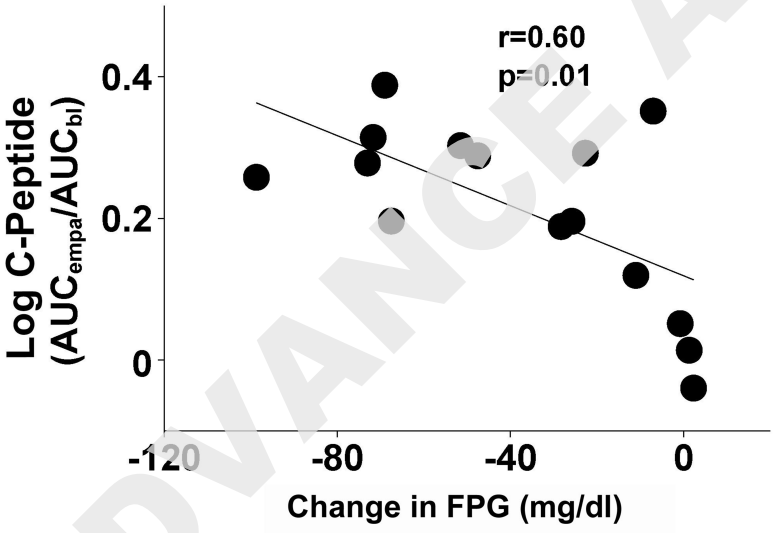

Figure 3

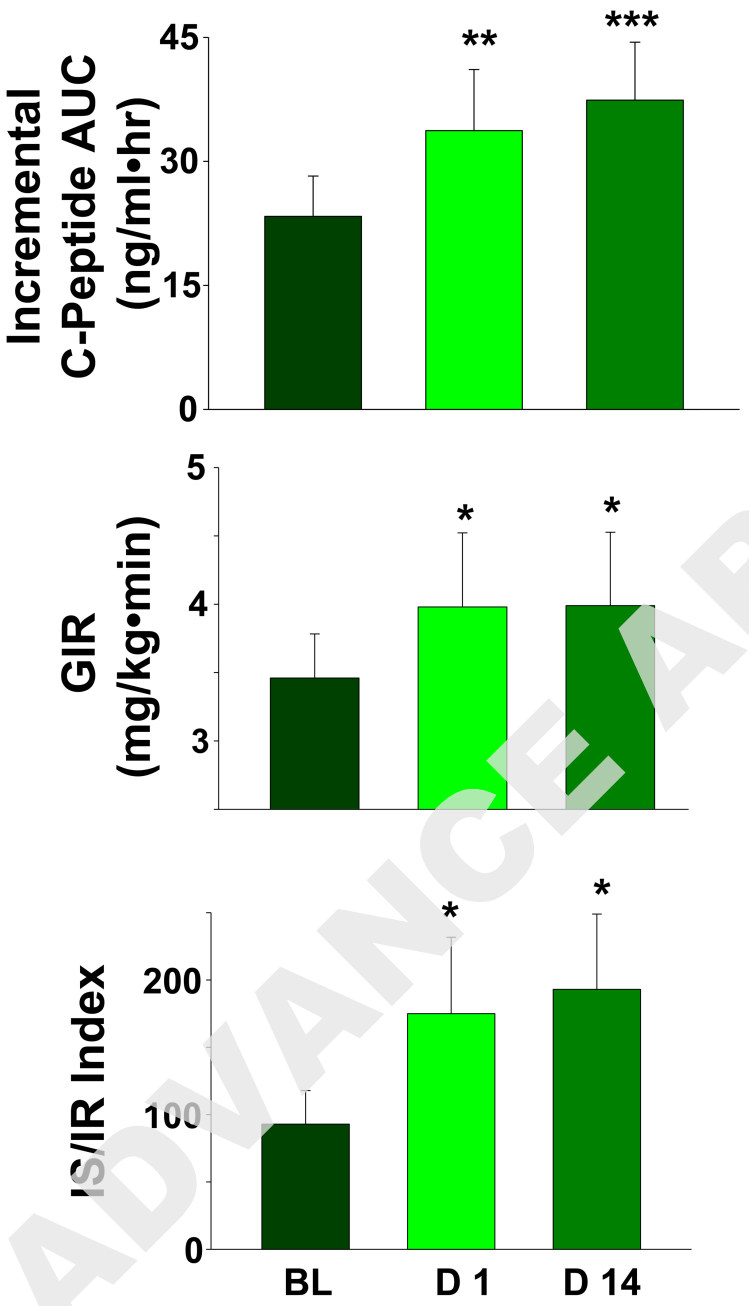

Figure 4