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Hyperglycemia and Hepatic Insulin Resistance

Mild Physiologic Hyperglycemia Induces Hepatic Insulin Resistance in Healthy Normal Glucose Tolerant Subjects

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Context: Chronic hyperglycemia worsens skeletal muscle insulin resistance and beta cell function. However, the effect of sustained physiologic hyperglycemia on hepatic insulin sensitivity is not clear.
Objective: To examine the effect of sustained physiologic hyperglycemia (similar to that observed in type 2 diabetic individuals) on endogenous (primarily reflects hepatic) glucose production (EGP) in healthy individuals.
Design: Subjects participated in a three step hyperinsulinemic (+10, +20, +40 mU/m^2·min) euglycemic clamp before and after a 48 hour glucose infusion to increase plasma glucose concentration by ~40 mg/dl above baseline. Endogenous glucose production (EGP) was measured with 3-^{3}H-glucose before and after chronic glucose infusion.
Participants: Sixteen NGT subjects (8 with and 8 without family history [FH] of diabetes) participated in the study.
Main outcome measure- EGP
Results: Basal EGP increased following 48 hours of glucose infusion (2.04±0.08 to 3.06±0.29 mg/kg_{ffm}•min, p<0.005). The hepatic insulin resistance index (basal EGP X fasting plasma insulin) markedly increased following glucose infusion (20.1±1.8 to 51.7± 6.6, p <0.005) in both FH+ and FH- subjects.
Conclusion: Sustained physiologic hyperglycemia for as little as 48 hours increased the rate of basal hepatic glucose production and induced hepatic insulin resistance in NGT healthy subjects, providing evidence for the role of glucotoxicity in the increase in hepatic glucose production in T2DM.

We examined the effect of mild physiologic hyperglycemia (+40 mg/dl) for 48 hours in healthy glucose tolerant subjects with and without family history of type 2 diabetes. .

INTRODUCTION

Postabsorptive hyperglycemia primarily reflects increased endogenous (hepatic) glucose production (EGP) and is a characteristic feature of type 2 diabetic patients with elevated fasting plasma glucose concentrations (> 140-160 mg/dl) (1-5) Glycogenolysis and gluconeogenesis contribute approximately equally to endogenous glucose production in normal glucose tolerant subjects (6-8). In poorly controlled T2DM individuals postabsorptive hyperglycemia primarily results from elevated hepatic gluconeogenesis (8-12). Although an absolute increase in EGP may not be observed in diabetic patients with mild fasting hyperglycemia, hepatic insulin resistance is evident by the impaired suppression of EGP by insulin (5,13-15). In pre-diabetic individuals elevated gluconeogenesis in the fasting state and impaired suppression of both gluconeogenesis and glycogenolysis by insulin (16) indicates that these metabolic defects are present early in the natural history of T2DM.

Chronic hyperglycemia has been shown to exacerbate skeletal muscle insulin resistance and normalization of the plasma glucose concentration leads to improved skeletal muscle glucose uptake (17-19). In animal models of T2DM, correction of hyperglycemia has been shown to normalize hepatic insulin sensitivity (17,20,21). Multiple factors have been suggested to contribute to the development of hepatic insulin resistance including lipotoxicity (22,23) and glucotoxicity (24,25) in T2DM. Increased hexosamine levels have been proposed as a possible mechanism responsible for the hepatic insulin resistance (26,27). Consistent with this, glucosamine infusion has been shown to induce hepatic insulin resistance (28) and in diabetic animal models lowering the blood glucose concentration by pharmacologic intervention improves hepatic insulin resistance (17, 18). In cultured hepatocytes both glucose and glucosamine (26,29) upregulate glucose-6-phosphatase via O-glycosylation of FoxO1 (26,28,30). These studies in rodents suggest that glucotoxicity plays an important role in the development of hepatic insulin resistance.

Although acute hyperglycemia is known to suppress endogenous glucose production (31-33), the effect of chronic hyperglycemia on hepatic insulin sensitivity in humans has not previously been examined. In the present study we examined the effect of sustained physiologic hyperglycemia, as seen in individuals with mild T2DM, on basal hepatic glucose production and suppression of HGP by insulin in normal-glucose-tolerant (NGT) subjects with and without FH of T2DM. Individuals with a positive family history (FH) of diabetes were included because we previously have shown that they are predisposed to the adverse effects of metabolic signals known to contribute to the development of T2DM, specifically lipotoxicity (34).

SUBJECTS AND METHODS

Subjects:

Eight healthy NGT subjects with FH of T2DM and 8 healthy NGT subjects without FH of T2DM participated in this study. Their clinical characteristics are shown in Table 1. No subject was taking any medication known to affect glucose metabolism. All subjects were in good general health as determined by medical history, physical exam, screening blood 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 an excessively heavy exercise program. Positive family history was defined as at least two first degree family members with T2DM. All studies were performed on the Bartter Research Unit (BRU), South Texas Veterans Health Care System, Audie L. Murphy Hospital in San Antonio, Texas.

All subjects received 75 gram OGTT to document the presence of normal glucose tolerance according to ADA criteria. Blood samples were drawn at -30, -15, 0 and every 15 minutes thereafter for 2 hours for determination of plasma glucose, insulin, C-peptide, and FFA concentrations. Body weight (nearest 0.1 kg on digital scale) (Health-O-Meter, Bridgeview, IL) and height (nearest 1 cm) were recorded. Total body fat content and percent body fat were measured by DXA (4500 Hologic, Bedford, MA).

Experimental Protocol:

All subjects received a baseline 3-step euglycemic insulin clamp (+10,+20, +40 mU/m^2●min) to raise plasma insulin by ~20, 40, 80 uU/ml (35) . Each step lasted for 90 minutes. The insulin clamp was performed at 7 AM following a ~10 hour overnight fast. At 10 PM on the evening prior to the insulin clamp, the first six subjects recruited into the study drank ^2H_2O, 1.67g/kg body water, in divided doses over 1 hour to quantitate gluconeogenesis. At 7 AM participants reported to the BRU and prime (40 uCi)-continuous (0.4 uCi/min) infusion of 3-^{3}H-glucose was started and continued throughout the 270 minute study to quantitate HGP. Blood samples for determination of C5 and C2 deuterated glucose enrichment were collected at -120, 0, 90, 180 and 270 minutes. Plasma samples for determination of plasma tritiated glucose specific activity were obtained at -30, -20, -10, -5, 0 minutes and every 10-15 minutes during the three step hyperinsulinemic euglycemic clamp to calculate the rate of hepatic glucose production, %HGP from gluconeogenesis, and total gluconeogenesis rate (6,9,10,11,36). Subjects who received ^2H_2O returned to the BRU in ~6 weeks (allowing for washout of ^2H_2O) and received a variable glucose infusion to raise the plasma glucose concentration by ~40 mg/dl for 48 hours. Subjects who did not receive ^2H_2O returned to the BRU within 3-5 days for the 48 hours glucose infusion. On the morning of day 3, the 3-step euglycemic insulin clamp with 3-^{3}H-glucose and ^2H_2O was repeated exactly as described above. The cold glucose infusion was stopped at 6 AM on the morning of day 3 and the plasma glucose concentration was allowed to drop spontaneously to the fasting level. At 8 AM the 3-step euglycemic insulin clamp was repeated.

Analytical Measurements:

Plasma glucose concentration was determined by the glucose oxidase reaction (Glucose Oxidase Analyzer; Beckman, Fullerton, CA), plasma insulin and C-peptide concentrations were measured by radioimmunoassay (Coat A Coat; Diagnostic Products, Los Angeles, CA), and plasma glucagon concentration was measured by radioimmunoassay (Linco Research, St. Charles, MO). Plasma 3-^{3}H-glucose radioactivity was measured in Somogyi precipitates. Enrichment of deuterium in carbons 2 and 5 of plasma glucose was measured as previously described (6,9,10,11,36).

STATISTICAL ANALYSIS:

Data are presented as mean ± SEM. Comparison of variables before and after 48 hours of glucose infusion was performed with analysis of variance (ANOVA) and Bonferroni post hoc correction. Correlation analysis was done with Pearson correlation coefficient. P values less than 0.05 were considered statistically significant.

Calculations:

Under steady-state post-absorptive conditions, the basal rate of endogenous glucose appearance (R_a) equals the 3-^{3}H-glucose infusion rate divided by steady-state plasma tritiated glucose specific activity. During the insulin clamp, nonsteady-state conditions for 3-^{3}H-glucose specific activity prevail and the rate of glucose appearance (R_a) was calculated with Steele's equation (37). The rate of residual EGP during the insulin clamp was calculated by subtracting the

exogenous glucose infusion rate from the tracer-derived R_a. The insulin-stimulated rate of total body glucose disposal (TGD) was calculated by adding the rate of residual EGP to the exogenous glucose infusion rate. Rates of gluconeogenesis were calculated by multiplying the plasma C5/C2 glucose ratio by the rate of endogenous glucose production. Glycogenolysis was calculated by subtracting the rate of gluconeogenesis from the rate of endogenous glucose production (6,9,10,11,36).

RESULTS

Plasma Glucose, Insulin, C-peptide and Glucagon Concentrations

Because the results in the FH- and FH+ are superimposable, we have combined the results for ease of presentation. Mean fasting glucose concentration was 97±2 mg/dl and during the 48 hour glucose infusion the plasma glucose concentration was maintained at 136 ± 4 mg/dl. The plasma insulin concentration during the 48 hour glucose infusion progressively increased from 10±1 uU/ml to 68±7 uU/ml ($p<0.005$). Before the start of the repeat euglycemic insulin clamp after 48 hours of sustained hyperglycemia, the plasma glucose concentration (135±5 mg/dl) was allowed to return to the fasting level (96± 3 mg/dl)l, at which time the plasma insulin concentration remained higher (17 ± 2 uU/ml, $p<0.05$) than on the day of the baseline insulin clamp. Of note, plasma glucose and insulin concentrations and plasma 3-^{3}H-glucose specific activity were at steady state prior to the start of the insulin clamp. Fasting plasma C-peptide levels followed a similar trend (2.8± 0.3 vs 1.86± 0.2 ng/ml, $p<0.05$) to those of insulin during the 48 hour glucose infusion. The steady state plasma insulin concentrations during the three insulin clamp steps were higher following 48 hours of glucose infusion (Figure 1A).

Plasma Glucose and Insulin Concentrations and Metabolic Clearance Rate of Insulin (MCRI) During the Insulin Clamp

The steady state plasma insulin (Figure 1A) and glucose concentrations during the 3 steps of the baseline insulin clamp were 25±1, 51±4, 87±3 uU/ml and 97±3, 98 ±2, 99 ±2 mg/dl, respectively. During the repeat insulin clamp performed after 48 hours of glucose infusion the steady state plasma insulin and glucose concentrations were 37±3, 67±5, 116±7 uU/ml and 99±2, 96 ± 2, 97 ±3 mg/dl. The increment in plasma insulin concentration during the 3 steps of the euglycemic insulin clamp performed before and after 48 hours of glucose infusion was significantly higher during the 40mU/m^2•min clamp step. The MCRI during the three insulin clamp steps (10, 20, and 40 mU/m^2.min) performed at baseline was 82±5, 66± 4, and 65± 3 ml/m^2.min respectively. Following 48 hours of sustained hyperglycemia, the MCRI was 71 ± 9 (P=NS), 60± 7 (P=NS), and 49 ± 4 ml/m^2.min ($p<0.05$ vs baseline).

Total Body Glucose Disposal

The rate of total body glucose disposal (TGD) during each of the three insulin clamp steps (10, 20 and 40 mU/m^2•min) was similar between FH+ and FH- subjects before and after 48 hours of glucose infusion. The rate of glucose disposal divided by the steady state plasma insulin concentration (TGD/SSPI) during the 40 mU/m^2•min insulin clamp step (Figure 2) was significantly reduced (6.71±0.79 vs 12.1±1.04 mg/kg$_{ffm}$ per uU/mL, $p<0.005$). Similar results were observed when the rate of glucose disposal was divided by the increment in steady state plasma insulin concentration. During the 40 mU/m^2•min insulin clamp step, TGD/ΔSSPI was 8.1±0.9 vs 14.7±1.2 mg/kg$_{ffm}$ per uU/mL, $p<0.005$).

Endogenous Glucose Production

The basal rate of EGP was similar in FH+ and FH- subjects during the baseline insulin clamp study. Following 48 hours of glucose infusion the basal rate of EGP increased similarly and significantly in the FH- (from 1.97± 0.1 to 3.5± 0.5 mg/kg•min, p<0.01) and FH+ (from 2.1± 0.1 to 2.55 ± 0.25 mg/kg•min, p<0.05) (Figure 3A) and in both groups combined (2.04±0.08 to 3.06±0.29 mg/kg$_m$•min, p<0.005) (Figure 1C). EGP declined progressively during each step of the euglycemic insulin clamp, with impaired suppression (p<0.01) observed during the 10 mU/m^2•min insulin clamp step (Figure 1B). Since the fasting plasma insulin concentration was significantly higher following 48 hours of glucose infusion, the hepatic insulin resistance index (basal EGP X fasting plasma insulin) increased markedly following glucose infusion in the FH- (16.8 ± 2.2 to 54.7±11, p<0.001) and FH+ (23±2.5 to 46±7.6, p<0.05) groups (Figure 3B) and in both groups combined (20.1±1.8 vs 51.7±6.6, p <0.001) (Figure 1C).

Gluconeogenesis and Glycogenolyis

The basal rate of gluconeogenesis (GNG) was measured by multiplying the ratio of C5/C2 in plasma glucose by the basal rate of EGP in 6 subjects. Following 48 hours of glucose infusion, the C5/C2 ratio tended to decrease from 0.54 ± 0.07 to 0.45 ± 0.04 (p=NS) and the basal rate of gluconeogenesis (5.58±1.8 vs 5.78±2.06 µmol/kg•min) did not change, while the basal rate of glycogenolysis increased significantly (8.02±1.7 to 10.5±1.3 µmol/kg•min, p<0.05). Thus, the increase in EGP and hepatic insulin resistance primarily resulted from impaired suppression of glycogenolysis.

Plasma Free Fatty Acid and Glucagon Concentrations

To gain insight into the mechanisms responsible for the development hepatic insulin resistance following 48 hours of glucose infusion, we measured plasma FFA and glucagon concentrations. Baseline plasma glucagon concentration tended to be higher following 48 hours of glucose infusion (64±3 vs 77± 8 pg/ml, p=0.25) (Figure 4), while the product of plasma glucagon and insulin concentrations was markedly increased (540 ± 56 vs 1336 ± 262, p=0.004) indicating that the suppressive effect of insulin on glucagon secretion was impaired. Baseline plasma free fatty acid concentration was markedly reduced following 48 hours of glucose infusion and suppressed to a similar magnitude during each of the 3 insulin clamp steps (Figure 4).

Plasma Lactate, Alanine and Glycerol

Plasma lactate, alanine, and glycerol are key gluconeogenic substrates. Although the plasma FFA was markedly suppressed following 48 hours of glucose infusion, there was no difference in plasma glycerol concentration (13.4±1.2 vs 13.3 ± 2.2 mg/L, p=ns). Following 48 hours of glucose infusion, there were small increases in both the plasma lactate (1.05± 0.05 vs 1.48 ± 0.07 mM, p<0.05) and alanine (2.38±0.3 vs 3.32±0.3 mM, p=0.03) concentrations.

Discussion

The present results demonstrate, for the first time, that mild sustained physiologic hyperglycemia for only 48 hours results in severe hepatic insulin resistance and an increase in the basal rate of endogenous (primarily reflects hepatic) glucose production. This observation has important clinical implications and indicates that mild persistent hyperglycemia aggravates hepatic insulin resistance and can exacerbate fasting hyperglycemia in T2DM patients.

Studies from our lab (1-3) and others (4,5,8,10,15) have shown that fasting hyperglycemia (plasma glucose>140 mg/dl) primarily results from hepatic insulin resistance and an elevated basal rate of hepatic glucose production. An acute rise in plasma glucose concentration has been shown to suppress hepatic glucose production in healthy individuals (32, 38). In contrast, the

present results show that 48 hours of sustained physiologic hyperglycemia in NGT individuals causes marked hepatic insulin resistance, resulting in an increased rate of basal hepatic glucose production and impaired suppression of HGP by insulin (Figure 3). The major difference between the previous studies (32,38) and the current study is the duration of hyperglycemia. In the previous studies (32,38) hyperglycemia was maintained for only 6 hours, while in the current study hyperglycemia was maintained for 48 hours. We did not examine the effect of acute hyperglycemia on EGP, so the present findings do not contradict these prior results. In healthy individuals acute hyperglycemia-induced suppression of EGP has been attributed, in part, to the glucose-induced reduction in plasma FFA concentration, secondary to suppression of lipolysis (39,40). In the present study, as expected, the plasma FFA concentration declined markedly following 48 hours of glucose infusion. This, if anything, would minimize (not increase) the severity of glucose-induced hepatic insulin resistance.

In NGT individuals approximately half of hepatic glucose production (HGP) is derived from glycogenolysis and half from gluconeogenesis (6-9, 11). In T2DM individuals the increased basal rate of HGP following an overnight fast primarily is derived from an elevated rate of hepatic gluconeogenesis (8-11). In the present study the percentage of glucose derived from gluconeogenesis (ratio of C5/C2 in glucose) was slightly, although not significantly, decreased after 48 hours of glucose infusion. When multiplied by the rate of basal HGP, the absolute rate of gluconeogenesis was not significantly changed. Thus, from the quantitative standpoint the increased rate of glycogenolysis was the primary factor responsible for the increased basal rate of HGP.

With respect to gluconeogenesis, substrate delivery to the liver has been shown to be an important determinant of hepatic gluconeogenesis (7). In the present study circulating levels of alanine and lactate increased slightly following 48 hours of glucose infusion, while plasma glycerol concentration was unchanged. Although the plasma alanine and lactate levels were increased following 48 hours of glucose infusion, the increase was quite small and, more importantly, the rate of gluconeogenesis was not increased. Free fatty acids are not a precursor for glucose but elevated plasma FFA levels can stimulate hepatic gluconeogenesis by activating enzymes involved in gluconeogenesis and stimulating glycogenolysis (22,41). However, in the present study plasma FFA levels were markedly decreased following glucose infusion for 48 hours.

Increase hexosamine flux has been shown to induce insulin resistance in muscle, adipocytes, and liver (27) by increasing intracellular levels of UDP-N-acetylglucosamine (UDP-GLc NAc), which subsequently is O-linked to serine/threonine residues of cytosolic proteins (42). This has been referred to as glucotoxicity (43). Although not measured in the present study, TRIB3 (44) has been suggested to be involved in glucose-mediated muscle insulin resistance. In rodent models of T2DM correction of hyperglycemia with a renal glucose transport inhibitor restores normal hepatic insulin sensitivity, reduces the elevated basal rate of hepatic gluconeogenesis, and inhibits glucose-6-phosphatase (17). Conversely, glucosamine infusion is normal rats induces hepatic insulin resistance and upregulates glucose-6-phosphatase (45). In cultured hepatocytes both glucose and glucosamine (45,46) upregulate glucose-6-phosphatase (G-6-Pase) via O-glycosylation of FOxO1 (46). When dephosphorylated, FOxO1 translocates to the nucleus and induces the transcription of key gluconeogenic genes which encode for PEPCK and G-6-Pase (47). Thus, increased hexosamine flux, as a result of chronic hyperglycemia, could induce insulin resistance via the FOxO1 pathway. Proof of this pathogenic mechanism would require liver biopsy before and after 48 hours of glucose infusion and this is not possible in humans.

Studies in rodents have demonstrated that sustained glucose infusion to create a state of physiologic hyperglycemia (plasma glucose concentration ~250 mg/dl) for 4 days resulted in greater than 90% suppression HGP (48). However, continued glucose infusion during days 5-7 was associated with a marked and progressive rise in HGP which was associated with an increase in plasma glucagon concentration. Thus, increased alpha cell glucagon secretion is a manifestation of glucotoxicity and contributes to the rise in HGP. In the present study the basal plasma glucagon concentration was increased by 20% (P=0.20) after 48 hours of glucose infusion. Given that the fasting plasma insulin concentration was increased following glucose infusion, the elevated plasma glucagon levels suggest resistance to the inhibitory effect of insulin on plasma glucagon. Consistent with this, the product of the plasma glucagon and plasma insulin concentrations was increased 2.5-fold following 48 hours of glucose infusion. Thus, increased plasma glucagon levels could have contributed, in part, to the increase in basal rate of HGP and impaired suppression during the euglycemic insulin clamp.

Although not measured in the present study, one would expect that 48 hours of glucose infusion would increase hepatic glycogen content (49,50) since acutely glucose binds to and activates glycogen synthase and inhibits glycogen phosphorylase (51,52). However, a chronic increase in hepatic glycogen concentration inhibits glycogen synthase and activates glycogen phosphorylase (53). Therefore, when glucose is infused for 48 hours, one could hypothesize that an increase in hepatic glycogen concentration could activate glycogen phosphorylase and accelerated the rate of glycogenolysis accounting, in part, for the present observations.

During the 48 hours of glucose infusion, insulin secretion was stimulated and the plasma insulin concentration increased significantly. We previously have shown that chronic hyperinsulinemia, while maintaining euglycemia, can induce hepatic, as well as peripheral tissue, insulin resistance (54,55). Insulin-induced insulin resistance is mediated via down regulation of the insulin signaling pathway (IR/IRS-1,2/PI3K/Akt) (56) and this could have contributed to the hepatic insulin resistance and elevated rate of basal HGP and impaired suppression of HGP by insulin (Figure 3). With regard to this, it should be noted that in T2DM patients combined hyperglycemia plus hyperinsulinemia commonly coexist, especially early in the natural history of T2DM, when beta cell function is still preserved (1).

Although not the primary purpose of the present study, 48 hours of sustained hyperglycemia was sufficient to induce peripheral tissue (primarily reflects muscle) insulin resistance. Thus, during the 40 mU/m^2•min insulin clamp step (Figure 2), insulin sensitivity (total glucose disposal ÷ steady state plasma insulin concentration) was reduced by 37%.

Although we hypothesized that NGT subjects with a positive family history of diabetes might be more susceptible to the deleterious effects of chronic hyperglycemia on hepatic insulin sensitivity, induction of hepatic insulin resistance and the increase in basal rate of HGP in FH positive subjects was similar to that in subjects without family history of diabetes.

In summary, sustained physiologic hyperglycemia (~40 mg/dl) for only 48 hours induced marked hepatic insulin resistance in NGT subjects with and without family history of diabetes.

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AUTHORS' CONTRIBUTION

All authors contributed to performing the study. The initial draft of the manuscript was prepared by DT, subsequently revised by RAD, and then reviewed and revised by all authors.

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I certify that neither I nor my co-authors have a conflict of interest as described above that is relevant to the subject matter or materials included in this Work

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Figure 1: **(A)** Plasma insulin concentration and before and during the three insulin clamp steps performed before (baseline) and after 48 hours of glucose infusion; **(B)** Hepatic glucose production during the three insulin clamp steps performed before and after 48 hours of glucose infusion; **(C)** Basal hepatic insulin resistance index (basal HGP X Fasting Plasma Insulin Concentration) before (baseline) and after 48 hours of glucose infusion

Figure-2: Insulin sensitivity (Total Glucose Disposal ÷ Steady State Plasma Insulin Concentration) during the 40 mU/m^2•min euglycemic insulin clamp performed before and after 48 hours of glucose infusion.

Figure-3: (A) Basal hepatic glucose production during the baseline study (solid bars) and during the study performed after 48 hours of glucose infusion (open bars) in the FH+ and FH- subjects; **(B)** Basal hepatic insulin resistance index (basal HGP X Fasting Plasma Insulin Concentration) before (solid bars) and after 48 hours of glucose infusion (open bars) in the FH+ and FH- subjects.

Figure- 4: (A) Plasma glucagon concentration and **(B)** plasma FFA concentration before and after 48 hours of glucose infusion.

Table 1. Baseline characteristics in NGT subjects without (FH-) and with (FH+) family history of diabetes. Data represent the mean ± SEM.

	FH-	FH+	P-Value
Number	8	8	
Gender (M/F)	5/3	4/4	ns
Age (years)	47±4	42±3	ns
BMI (kg/m^2)	27±1	25.5±1.1	ns
FFM (kg)	54±6.4	48±4.3	ns
HbA1c (%)	5.4±0.1	5.5±0.1	ns
FPG (mg/dl)	95±4	94±5	ns
2-h Glucose OGTT (mg/dl)	110±7	128±9	ns

BMI = body mass index; FFM = fat free mass; FPG = fasting plasma glucose concentration

Figure 1A

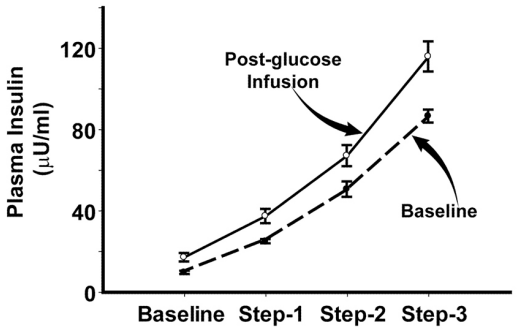

Figure 1B

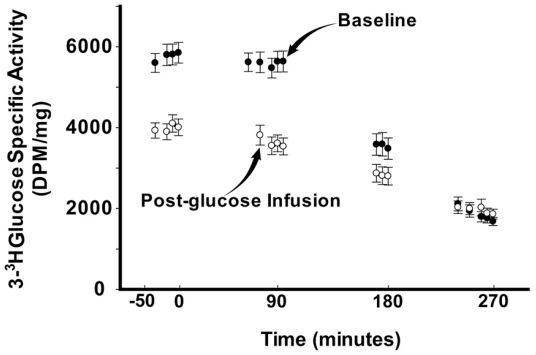

Figure 3C

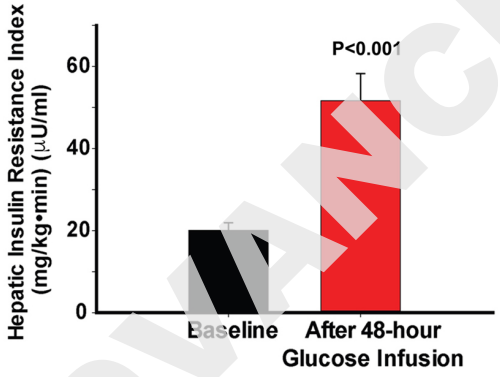

Figure 2

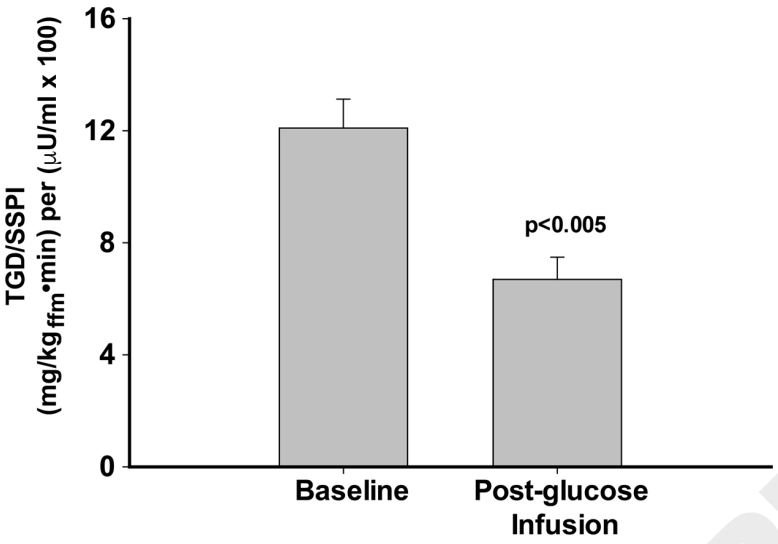

Figure 3A

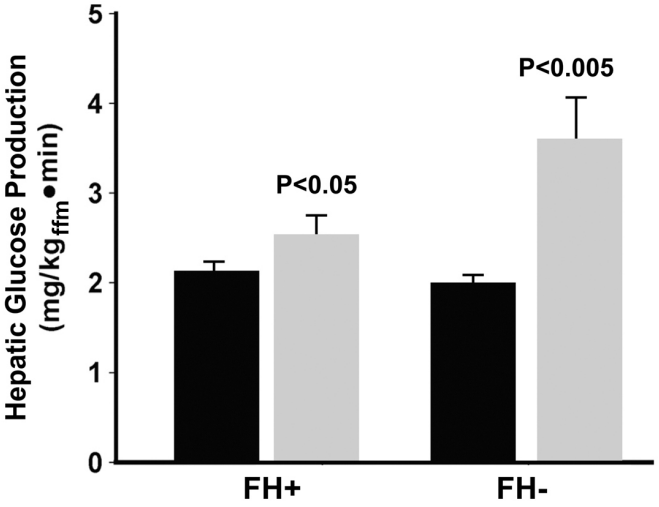

Figure 3B

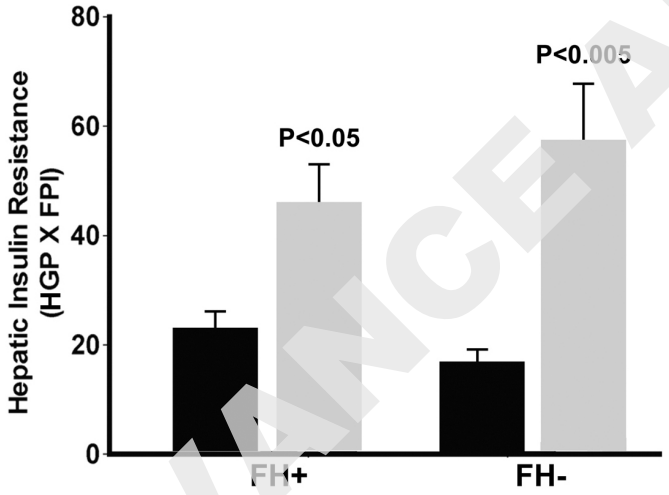

Figure 4A

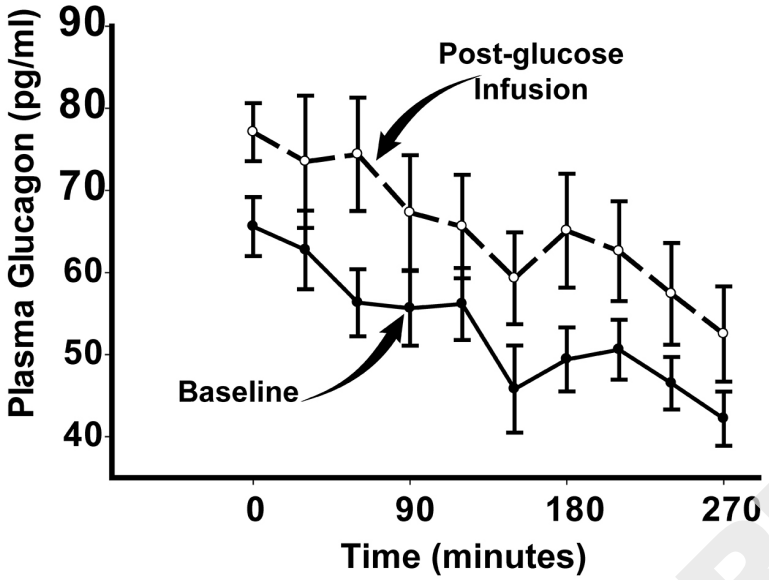

Figure 4B