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Obesity, type 2 diabetes, and impaired insulin stimulated blood flow: role of skeletal muscle NO synthase and endothelin-1

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Running title: Endothelial insulin signaling in type 2 diabetes

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Abstract

Increased endothelin-1 (ET-1) and reduced endothelial nitric oxide phosphorylation (peNOS) are hypothesized to reduce insulin-stimulated blood flow in type 2 diabetes (T2D), but studies examining these links in humans are limited. We sought to assess basal and insulin-stimulated endothelial signaling proteins (ET-1 and peNOS) in skeletal muscle from T2D patients. Ten obese T2D (glucose disposal rate (GDR): 6.6±1.6 mg/kg LBM/min) and eleven lean insulin sensitive subjects (Lean GDR: 12.9±1.2 mg/kg LBM/min), underwent a hyperinsulinemic-euglycemic clamp with vastus lateralis biopsies taken before and 60 min into the clamp. Basal biopsies were also taken in eleven medication- naïve, obese, non-T2D subjects. ET-1, peNOS (ser1177), and eNOS protein and mRNA were measured from skeletal muscle samples containing native microvessels. Femoral artery blood flow was assessed by duplex Doppler ultrasound. Insulin-stimulated blood flow was reduced in obese T2D (Lean: +50.7±6.5% baseline, T2D: +20.8±5.2% baseline, $p<0.05$). peNOS/eNOS content was higher in Lean under basal conditions and although not increased by insulin, remained higher in Lean during the insulin clamp than in obese T2D ($p<0.05$). ET-1 mRNA and peptide were 2.25±0.50 and 1.52±0.11 fold higher in obese T2D compared to Lean at baseline, and ET-1 peptide remained 2.02±1.9 fold elevated in obese T2D after insulin infusion ($p<0.05$), but did not increase with insulin in either group ($p>0.05$). Obese non-T2D subjects tended to also display elevated basal ET-1 ($p=0.06$). In summary, higher basal skeletal muscle expression of ET-1 and reduced peNOS/eNOS may contribute to a reduced insulin-stimulated leg blood flow response in obese T2D patients.

New and Noteworthy

Although impairments in endothelial signaling are hypothesized to reduce insulin-stimulated blood flow in type 2 diabetes (T2D), human studies examining these links are limited. We provide the first measures of nitric oxide synthase and endothelin-1 expression from skeletal muscle tissue containing native micro-vessels in individuals with and without T2D before and during insulin stimulation. Higher basal skeletal muscle expression of endothelin-1 and reduced peNOS/eNOS may contribute to reduced insulin-stimulated blood flow in obese T2D patients.

Introduction

It is estimated that one third of the U.S. population will have diabetes by 2050 (6). A key etiological factor in type 2 diabetes (T2D) is insulin resistance, which leads to both fasting and post-prandial hyperglycemia (41). Another negative outcome in T2D is the presence of vascular dysfunction (21, 26). Indeed, increasing evidence suggests that impaired vasodilation in response to insulin plays a key role in the development and progression of insulin resistance and cardiovascular disease (4, 48). We (31) and others (25) have found that individuals with T2D have a severely blunted insulin-mediated blood flow response. Although an impaired vasodilatory response to insulin has been established in T2D patients, the underlying mechanisms have not been rigorously examined.

Previous work has established that insulin-stimulated increases in blood flow play an important role in insulin-stimulated glucose uptake into skeletal muscle (20). Baron et al (3) demonstrated that leg glucose uptake during a hyperinsulinemic-euglycemic clamp was significantly reduced (~40%) when L-NAME, a nitric oxide synthase (NOS) inhibitor, was concurrently infused to reduce blood flow in healthy insulin sensitive subjects. These data indicate that the ability of insulin to stimulate the production of nitric oxide (NO) is important for insulin-stimulated glucose disposal. Insulin phosphorylates endothelial NOS (eNOS) stimulating the production of the vasodilator NO (39). The resulting vasodilation and subsequent increase in blood flow results in enhanced delivery of glucose and insulin to skeletal muscle (9). Interestingly, further evidence suggests that insulin also increases the production and release of endothelin-1 (ET-1), a potent vasoconstrictor. In healthy, insulin sensitive subjects, Ottosson et al. (36) demonstrated significant reductions in leg glucose uptake during a hyperinsulinemic-euglycemic clamp following ET-1 infusion, providing evidence that this pathway is involved in glucose control. Overall, the use of inhibitors of eNOS to block vasodilation or infusion of ET-1 to cause vasoconstriction has been shown to alter insulin-stimulated blood flow and reduce glucose uptake in healthy humans. Likewise, impaired endothelial insulin-stimulated signaling has been reported in insulin resistant rodent models (13, 23). However, the extent to which an imbalance in the production of NO and ET-1 contributes to the reduced insulin-stimulated blood flow found in patients with T2D has not been mechanistically tested (13,

39). We are unaware of *in-vivo* studies in which peNOS and ET-1 expression have been measured in the skeletal muscle of T2D patients before and during hyperinsulinemia.

Thus, the purpose of this study was to test the hypothesis that ET-1 content is increased and eNOS phosphorylation (ser1177) decreased in skeletal muscle microvasculature during insulin stimulation in obese individuals with T2D compared to healthy lean insulin sensitive (Lean) individuals. Given that previous studies have demonstrated that individuals across the insulin sensitivity/resistance continuum (Lean, obese, obese T2D) display a progressive impairment in insulin-stimulated blood flow responses (24), we chose to examine the broad ends of this continuum (i.e., Lean and obese T2D patients) to begin to examine potential impairments in endothelial signaling proteins in response to insulin stimulation. We reasoned that this approach would provide important mechanistic insight regarding the production of these signaling proteins in T2D, which may be responsible for decreased glucose uptake, and as such, provide support for the vasculature as a target for therapeutic intervention in patients with T2D. Further, we also compared basal levels of ET-1 and eNOS to age and body weight matched non-T2D obese individuals obtained from biopsy samples for a previously published study (30) from our laboratory to better understand the impact of obesity, independent of T2D, on our basal results.

Methods

Subjects

Protocols were approved by the University of Missouri Health Sciences Institutional Review board and written informed consent was obtained from all subjects. We recruited 21 individuals to complete this study [11 Lean individuals (4 females/7 males) and 10 obese T2D patients (7 female/ 3 male)]. T2D patients had a clinical diagnosis of T2D and all subjects completed a medical health history questionnaire and a 12-h fasting blood chemistry screening including a lipid panel and a metabolic panel that included insulin and glucose measurements. Additionally, muscle samples from obese-non-T2D individuals were also assessed (n=11; 6 female/ 5 male). Subject characteristics are provided in Table 1. Exclusion criteria included smoking, multiple daily injections of insulin (once daily insulin was allowed), recent weight gain or loss (>5% of body weight in 3 months), recent (<3 mo) changes in medication use or dose, uncontrolled T2D (HbA1c>10%), advanced retinopathy or neuropathy, pregnancy, known cardiovascular or pulmonary disease, consumption of > 14 alcoholic beverages per week (1) (Lean: 1.38±0.83 and T2D: 0.8±0.4 drinks/week), and individuals on prescription anticoagulants.

Experimental Procedures

Body composition was assessed via Dual X-ray Absorptiometry (QDR-4500A, Hologic, Shelby Township, and Michigan) prior to the study visit. Subjects were asked to refrain from vigorous physical activity and alcohol for 24 hours prior to the study visit. Twelve hours prior to the study visit subjects refrained from food and drink (other than water). The morning of the study, the subjects refrained from taking all medications until after the study visit was over.

Upon arrival to the lab, weight and height were measured. Subjects then rested in a supine position while intravenous catheters were inserted in a hand and antecubital vein for blood draws and glucose and insulin infusion, respectively. The lower portion of the arm used to obtain blood draws was placed in a warming box for arterialization of hand vein blood samples (54). After resting quietly for > 20 minutes, baseline blood samples were obtained for the analysis of insulin and glucose. A muscle sample from the vastus lateralis was then taken using a Bergstrom needle and standard biopsy techniques as

previously described by our lab (30, 49). Briefly, approximately 60 to 120 mg of skeletal muscle tissue, and associated micro-vessels perfusing the muscle, was taken from the vastus lateralis. Connective and adipose tissue were removed from the muscle sample and the samples were snap frozen in liquid nitrogen and stored at -80˚C until analyzed. After another 30 minutes rest, a hyperinsulinemic-euglycemic clamp ($40\mu U/m^2/min$) was started (60), with blood glucose samples taken every 5 minutes for the determination of glucose infusion rate. Further, every 30 min blood was drawn into serum separator tubes, spun, and stored at -80˚C for later insulin and glucose analysis. Given the range of insulin sensitivities, some individuals took longer to obtain a steady glucose infusion rate, which lead to a 2-3 hour range of the insulin clamps. Common femoral artery blood flow was measured via duplex Doppler ultrasound (Logiq P5, GE Medical Systems, Milwaukee, WI) at baseline, ~20 minutes after the baseline muscle biopsy and at minute 45 of the insulin clamp using a 11-Mhz linear array transducer as previously described by our lab (31, 43, 52). Blood flow ($mL\ min^{-1}$) was calculated as: blood flow= π x $(diameter/2)^2$ x V_{mean} x 60, where V_{mean}= mean velocity. Sixty minutes into the hyperinsulinemic-euglycemic clamp a second muscle biopsy was taken as described above. Glucose disposal rate was calculated during the last 45 minutes of the hyperinsulinemic-euglycemic clamp. A hyperinsulinemic-euglycemic clamp was not performed in 1 obese T2D due to an inability to obtain IV access and blood flow was not measured in 1 subject due to technical difficulties. Further, due to issues related to blood pressure cuff interference with the IV, blood pressure at baseline and minute 45 of the hyperinsulinemic-euglycemic clamp was only collected in 5 Lean and 4 obese T2D subjects.

Tissue Analysis

Skeletal muscle tissue containing native micro-vessels was assessed for eNOS, peNOS, AKT, and pAKT protein and ET-1 peptide via standard western blotting procedures as performed previously (32). Additional muscle samples from non-T2D obese subjects were also assessed for ET-1 and eNOS under basal conditions and compared to the Lean samples and obese T2D samples. eNOS is not expressed in skeletal muscle (10, 46), and thus, any protein content that was measured would be from micro-vessels only. Further, to date, no studies have confirmed that ET-1 is produced from skeletal muscle (18). In

144 contrast, AKT is in muscle tissue and micro-vessels. eNOS and peNOS antibodies were purchased from
145 BD transduction (catalogue number: 610296 and 612392, respectively) and ET-1 from Sigma Aldrich
146 (catalogue number: e166). Briefly, 20 µg of protein homogenate (2 µg/µL) was loaded on 7.5%
147 (eNOS/peNOS and AKT/pAKT) and 15% (ET-1) gels, and transferred to membrane. After blocking for
148 an hour in 5% nonfat milk, membranes were incubated overnight in 5% BSA and the respective primary
149 antibody (1:1000). Following the overnight incubation samples were incubated in 5% milk and their
150 respective secondary antibodies (1:5000) for 1 hour and imaged. A total protein stain was used for the
151 loading control as performed previously (2). The blots were stained with 0.1% Napthol blue black
152 bioreagent (Sigma Aldrich) and washed in 10% acetic acid. Total band intensity for each lane (not
153 including the band of interest) was then used as the loading control for each lane. Importantly, we have
154 tested other ET-1 antibodies from other sources and although they showed bands at the correct molecular
155 weight they also had non-specific bands. However, the ET-1 antibody from Sigma that we utilized
156 showed a clear band at the correct MW range of 24-30 kD.

157 Skeletal muscle tissue containing native micro-vessels was also assessed for NOS3 (eNOS), and
158 pre-pro ET-1 mRNA expression via real time PCR as previously described (16, 37). Briefly, frozen
159 skeletal muscle samples were homogenized in TRIzol solution using a tissue homogenizer (TissueLyser
160 LT, Qiagen, Valencia, CA). The Qiagen's RNeasy tissue protocol was used to isolate total RNA.
161 Samples were Nanodroped (Thermo Scientific, Wilmington, DE) to determine RNA concentration and
162 purity. Total RNA was made into first-strand cDNA via the High Capacity cDNA Reverse Transcription
163 kit (Applied Biosystems, Carlsbad, CA). The ABI StepOne Plus sequence detection system (Applied
164 Biosystems) was used to run quantitative real-time PCR with primer sequences developed from the NCBI
165 Primer Design tool and purchased from IDT (Coralville, IA). The primer sequences were as follows:
166 Endothelin 1: sense 5'-CAGAAACAGCAGTCTTAGGCG-3', antisense 5'-
167 GGTGGCAGAAGTAGACACACT-3'; NOS3: sense 5'-ATCCCCCGGAGAATGGAGAG-3', antisense
168 5'-AGTGGGTCTGAGCAGGAGAT-3'; 18S: sense 5'-ATACAGCCAGGTCCTAGCCA-3', antisense
169 5'-AAGTGACGCAGCCCTCTATG-3'. A 20 µL reaction mixture containing 10 µL iTaq UniverSYBR

Green SMX (BioRad, Hercules, CA) and the appropriate concentrations of gene-specific primers plus 4 µL of cDNA template were loaded in a 96-well plate. Samples were run in duplicate as described: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 45 s. The specificity of PCR primers was verified by a dissociation melt curve. 18S was used as house-keeping control gene. 18S cycle threshold (CT) was not different between the groups and between basal and insulin stimulation conditions. mRNA expression values are presented as $2^{\Delta CT}$. ΔCT = 18S CT - gene of interest CT. mRNA levels were normalized to the Lean group under basal conditions. Due to lack of sample, insulin-stimulated skeletal muscle eNOS and ET-1 mRNA were not analyzed in 3 Lean subjects.

Blood Analysis

Serum samples during the hyperinsulinemic-euglycemic clamp were analyzed for glucose via the glucose oxidase method and insulin via enzyme-linked immunosorbent assays (Immulite 1000 Analyzer, Siemens, Deerfield, IL) as performed previously (31).

Statistical Analysis

A power analysis was performed on preliminary data generated from our laboratory on peak blood flow responses during the insulin clamp in sedentary individuals with T2D compared to healthy controls. The analysis was conducted with β=0.20m, power=0.8 and α=0.08. From the power analysis it was determined that 8 subjects would allow us to reach statistical significance for insulin stimulated blood flow responses. However, there is a lack of data on endothelial signaling proteins in T2D humans during an insulin clamp, which alludes to the novel nature of this study. Thus, we also increased our sample size (11 lean and 10 obese T2D) to account for this discrepancy.

Glucose disposal rate, percent change in blood flow from baseline to minute 45 of the clamp, BMI, percent body fat, age, fasting glucose, total cholesterol, triglycerides and HOMA-IR between the Lean individuals and obese T2D individuals were assessed by unpaired student's t-tests. A 2 way group x time repeated measures ANOVA was used to assess eNOS, peNOS/eNOS, AKT, pAKT/AKT protein and eNOS, and ET-1 mRNA expression from basal to 60 minutes of insulin stimulation in the Lean individuals and obese T2D individuals. A one way ANOVA was used to assess basal levels of eNOS,

peNOS/eNOS, AKT and pAKT/AKT protein between the Lean individuals, obese individuals, and obese T2D individuals. Sex was not considered a factor in the statistical analysis of the data. Data are presented as mean ± S.E. Statistical significance was accepted at α of $p < 0.05$.

Results

Subject Characteristics are displayed in Table 1. Subject medications are listed in Table 2.

Glucose homeostasis - Insulin Sensitivity

Fasting blood glucose and HOMA-IR were significantly higher in obese T2D individuals compared to Lean individuals and obese non-T2D subjects (Table 1) ($P<0.001$). Glucose and insulin levels during the hyperinsulinemic-euglycemic clamp were higher in the obese T2D individuals compared to the Lean individuals (Figure 1 A and B) ($p<0.001$ and $p=0.04$, respectively) an effect that occurs, in part, because of higher initial glycaemia and insulin levels, and decreased clearance of both in the T2D condition. Glucose disposal rate during the hyperinsulinemic-euglycemic clamp was also significantly lower in the obese T2D individuals compared to the Lean individuals, as expected (Table 1) ($p=0.007$).

Femoral Artery Blood Flow

Baseline blood flow before and approximately 15-20 minutes following the muscle biopsy was found to be unchanged in Lean individuals and obese T2D individuals (Lean: pre: 252.3±20.9, post: 299.4±23.9 ml/min, $p=0.2$; obese T2D: pre: 264.9±40.0, post: 261.2±41.9 ml/min, $p=0.9$). There was a significant effect of insulin to increase blood flow within each group ($p<0.001$) with the percent change in femoral artery blood flow from baseline to 45 min of the hyperinsulinemic-euglycemic clamp greater in the Lean individuals compared to the obese T2D individuals ($p=0.004$) (Figure 1C). Mean arterial pressure during the hyperinsulinemic-euglycemic clamp was not altered (T2D: baseline: 96±5 vs. clamp: 96±4, $p=0.94$; Lean: baseline: 83±4 vs. clamp: 84±5 mmHg; $p=0.91$); however, obese T2D had higher basal blood pressure than the Lean ($p=0.05$). Femoral artery diameter was unchanged during the hyperinsulinemic-euglycemic clamp and was not different between the Lean individuals and obese T2D individuals (Lean: baseline: 0.88±0.8 vs. clamp: 0.90±0.8 cm; $p=0.69$; T2D: baseline: 0.78±0.1 vs. clamp: 0.77±0.1 cm, $p=0.95$).

Skeletal muscle tissue analysis

There was a significant main effect for the obese T2D group to have higher pre-pro ET-1 mRNA (Figure 3B, $p=0.04$) and ET-1 peptide (Figure 2E, $p<0.001$) compared to the Lean group. No effect of

insulin (ET-1 peptide, p=0.242; ET-1 mRNA, p=0.207) or interaction (ET-1 peptide, p=0.180; ET-1 mRNA, p=0.215) was found between the two groups and as such, ET-1 remained higher in the obese T2D group during the hyperinsulinemic-euglycemic clamp. No significant differences were demonstrated in NOS3 mRNA (Figure 3A) between Lean individuals or obese T2D individuals (p=0.193); however there was a main effect for the obese T2D group to have higher eNOS (Figure 2C, p=0.026) but lower peNOS/eNOS (Figure 2D, p=0.003) protein compared to the Lean individuals. No main effect of insulin (p=0.664) or interaction (p=0.357) was demonstrated between the two groups such that peNOS/eNOS remained lower in the obese T2D group during the hyperinsulinemic-euglycemic clamp.

There was a main effect for the obese T2D group to have a slightly higher total AKT (Figure 2A, p=0.05)). No main effect of insulin (p=0.294) or interaction (p=0.847) on total AKT was demonstrated between the two groups. While both the Lean individuals and the obese T2D individuals had an increase in pAKT/AKT (Figure 2B, p<0.001)) to insulin stimulation, pAKT/AKT increased to a greater extent in the Lean individuals (p=0.004) confirming reduced insulin signaling in skeletal muscle of obese T2D subjects compared to Lean.

Because we observed basal, non-insulin-stimulated differences in endothelial signaling proteins between the Lean individuals and obese T2D individuals, we also selected skeletal muscle biopsy samples from a previously published study (30) from 11 obese non-T2D subjects who were age and BMI matched to the obese T2D in this investigation to determine the specific role of T2D independent of obesity alone. The characteristics of the obese-non-T2D individuals are presented in Table 1. No significant basal differences existed between Lean individuals, obese individuals, and obese T2D individuals in AKT (Figure 4A, p=0.388), pAKT/AKT (Figure 4B, p=0.670), or eNOS (Figure 4C, p=0.288) protein. However, peNOS/eNOS (Figure 4E) protein trended (p=0.054) to be significantly different between the three groups. There was also a trend for statistical significance for ET-1 peptide content to be elevated in the obese individuals and obese T2D individuals compared to Lean individuals (Figure 4D) (p=0.064).

Insulin sensitivity and endothelin-1 expression correlations

GDR was not significantly correlated to ET-1 peptide or mRNA (r= -0.240, p=0.2 and r=-0.335, p=0.4, respectively). Also, HOMA-IR was not correlated to ET-1 peptide or mRNA (r=-0.08, p=0.7 and r=-0.22, p=0.3, respectively). However, fasting blood glucose trended to be significantly correlated to ET-1 peptide (r=0.374, p=0.09) and was significantly correlated to ET-1 mRNA (r=0.518, p=0.02).

Discussion

Insulin stimulation did not alter skeletal muscle peNOS/eNOS, eNOS or ET-1 peptide content or mRNA expression in either Lean individuals or obese T2D individuals, despite the Lean showing a greater than 50% higher insulin-stimulated blood flow and ~2 fold higher pAKT/AKT signaling than the obese T2D. The obese T2D individuals displayed significantly higher basal protein content and gene expression of the potent vasoconstrictor ET-1 and lower peNOS/eNOS protein expression, compared to the Lean individuals. Overall, these results indicate that 1 hour of hyperinsulinemia with insulin levels similar to that of post-prandial concentrations (31, 43), does not significantly alter eNOS or ET-1 expression in Lean individuals or obese T2D individuals. However, higher basal levels of ET-1 and reduced peNOS/eNOS may contribute to a reduced insulin-stimulated blood flow response in obese T2D patients.

Contrary to our hypothesis, 60 min of insulin stimulation caused no significant changes in peNOS/eNOS or ET-1 expression in the skeletal muscle homogenate of either the Lean or obese T2D. This occurred despite increased pAKT in the muscle at this same time in both groups. Cell culture studies of human umbilical endothelial artery cells and bovine aortic endothelial cells have shown significant increases in peNOS (56) and ET-1 mRNA expression (35) following 30 and 60 minutes of insulin stimulation. Since prior research demonstrated increases in leg blood flow to insulin stimulation or glucose ingestion at 45-60 minutes (31) we had speculated that peNOS/eNOS and ET-1 expression in the present study would also be elevated or altered at this time and would show differential responses between Lean individuals and obese T2D. However, this was not found. Nevertheless, it should be mentioned that there was a trend for ET-1 peptide to increase following insulin stimulation in the T2D condition, but this was not statistically significant ($p=0.18$) and future studies are necessary to confirm this. Moreover, ET-1 mRNA did not increase following insulin stimulation in the T2D suggesting that there was no increase in the transcription of ET-1 with insulin.

Our findings of a lack of insulin stimulated activation of peNOS or changes in ET-1 may be different than previous studies for several reasons. First, the insulin levels used in the previous cell culture

studies were supraphysiological and likely providing a greater stimulus for increased peNOS and ET-1 expression than postprandial levels used in the current study. Further, other cell culture data supports that peNOS is increased within 5 minutes of insulin stimulation (42), so it is possible in the present study that peNOS increased acutely to insulin stimulation then returned towards basal levels following prolonged stimulation. There is also some evidence that supports that prolonged agonist exposure leads to inactivation of eNOS (12, 29). However, if this occurs with prolonged insulin stimulation and/or the time course by which eNOS is inactivated is not known. Protein phosphatase 2A (PP2A) regulates the dephosphorylation of Ser 1177 on eNOS which would inactivate eNOS (12) but we are not aware of any time-course studies examining the activity of PP2A to insulin stimulation. Future studies need to examine the *in-vivo* time course of peNOS activation and activation of inhibitors of eNOS to determine how these are altered with prolonged insulin stimulation at postprandial concentrations.

While we and others demonstrate an increase in leg blood flow following 45-60 minutes of insulin stimulation (31), others report a more variable time of maximal blood flow responses (8, 24). This may be due to differences in insulin sensitivities, insulin dose and concentration, and duration of exposure. Individuals with insulin resistance have greater plasma insulin responses to meals that also remains elevated for a longer period of time following a meal when compared to lean insulin sensitive individuals. Thus, blood flow (and endothelial signaling proteins) between the lean and the obese T2D individuals may be altered differently following longer periods of insulin stimulation than the 1 hr used in the present study. Indeed, previous reports by Kashyap et al. (17) demonstrate in non-T2D subjects that significant increases in eNOS expression are induced following 4 hours of insulin stimulation, while this duration of insulin infusion did not change eNOS in T2D subjects. However, *in-vitro* studies in endothelial cells indicate that insulin stimulated eNOS phosphorylation occurs at the onset of insulin exposure (42) and this is rapidly changed back to basal levels through a putative feedback control system (12, 29). We measured eNOS and ET-1 in the microvessels of skeletal muscle biopsies at 1 hour, a time-point when our preliminary studies demonstrated that insulin infusion caused a significant increase in blood flow compared to baseline. However, eNOS phosphorylation was not increased with 1 hour of

insulin infusion. Given the already previously mentioned findings, it is possible that either a shorter or longer duration of insulin infusions would have revealed a significant change in peNOS in either of the groups. Future studies need to address this.

In the present study, obese T2D individuals had significantly elevated basal eNOS protein content compared to the Lean individuals but the obese T2D individuals did not express greater peNOS. This is in contrast to previous reports comparing weight matched controls to T2D patients that reported decreased (53) and no differences in basal eNOS (17) expression in T2D patients. The greater elevations of eNOS protein content in the obese T2D individuals in the present study may be due in part to chronic greater basal and post-prandial insulin concentrations, a stimulus known to increase eNOS content (22). Thus, it is possible that the elevation in eNOS in obese T2D is a compensatory mechanism to sustain similar amounts of peNOS compared to non-T2D individuals. Aged rodents (55) and humans (11) also display increased eNOS expression which is postulated to counter the reduced NO bioavailability and NO mediated vasodilation shown in aged population, thus it is possible the same phenomenon occurs with obesity and T2D.

As previously stated, a key finding was that the obese T2D showed significantly higher basal peptide content of the potent vasoconstrictor ET-1 than the Lean subjects. Likewise, previous studies have demonstrated elevated basal plasma levels of ET-1 in individuals with T2D compared to controls (45). Since ~80% of endothelin-1 is secreted on the abluminal side and putatively signals locally through a paracrine fashion (58), plasma ET-1 is not considered the best marker of cellular levels of endothelial ET-1 concentrations. Thus, in this regard, direct measures of ET-1 in the vasculature of skeletal muscle homogenate provide an advantage over blood samples. Importantly, previous studies have demonstrated that ET-1 blockade significantly increased blood flow to a greater extent in individuals with T2D compared to non T2D individuals (27, 28, 47, 50) suggesting that individuals with T2D have greater ET-1 mediated basal vasoconstrictor tone. These studies, and others (7, 40, 44, 51), demonstrate the important role of obesity and insulin resistance in altering ET-1 mediated vasoconstriction. Therefore, the greater basal ET-1 content found in this study and associated vasoconstriction, combined with previous inhibitor

studies, suggests that elevated ET-1 may partially explain the blunted insulin-stimulated blood flow response in the obese T2D individuals.

Since we demonstrated significant elevations in basal levels of ET-1 and eNOS in the obese T2D compared to the Lean individuals, we were interested in understanding if these effects were due to the pathology of T2D or were caused by the obese state or age differences in our Lean subjects and obese T2D subjects. Thus, we examined ET-1 peptide from skeletal muscle biopsy samples collected from a previously published paper (30) in subjects that were age and BMI matched to the obese T2D subjects in the current study. These data showed that obese and obese T2D have similar ET-1 peptide levels, alluding to the possibility that elevated ET-1 levels in the vasculature occur during the obese state and preclude the development of T2D. Similarly, Kim et al. (19) showed that high fat fed, obese mice displayed insulin resistance in the vasculature before skeletal muscle, liver, or adipose tissue suggesting that impairments in the vasculature occur early in the development of insulin resistance. Further, work from our group (33) has demonstrated that sedentary obese, insulin resistant OLETF rats, prior to the development of T2D, have greater insulin-stimulated vasodilation to ET-1 blockade compared to their sedentary insulin sensitive counterparts, providing further evidence that up regulation of ET-1 occurs early in the progression of insulin resistance and likely prior to T2D.

Obesity has been shown to result in increased ET-1 peptide and prepro-ET-1 gene expression (5, 34, 57). Thus, it is likely that obesity (and hyperglycemia) are both driving the trend for higher ET-1 expression in the obese and obese T2D compared to Lean. Endothelin-1 expression increases in endothelial cells exposed to hyperglycemic conditions (14, 59). Correlational analysis revealed that fasting blood glucose was significantly correlated to ET-1 mRNA and trended to be correlated to ET-1 peptide, further suggesting a link between glucose and ET-1 expression. Given that the obese T2D individuals were on antidiabetic medications to treat hyperglycemia, ET-1 expression may be reduced compared to an obese T2D that is not treated. Insulin also increases endothelin-1 via MAPK mediated pathways (15, 38). Thus, chronic fasting hyperinsulinemia is likely another important contributor of the

increased basal ET-1 in obese T2D compared to Lean subjects, however, as already stated, we did not see evidence that acute insulin stimulation increases ET-1 mRNA or peptide in either subject group.

To summarize, we demonstrated for the first time in humans that skeletal muscle phosphorylation of eNOS does not significantly increase following 1 hour of post-prandial levels of insulin in either Lean individuals or obese T2D individuals. However, significant increases in peNOS/eNOS were found in Lean individuals compared to obese T2D individuals in the basal, non-insulin-stimulated state. Further, basal, non-insulin-stimulated levels of ET-1 were elevated in obese T2D individuals, but were not altered with 60 minutes of insulin stimulation. Additionally, we provide evidence that elevated ET-1 peptide may occur in obesity prior to the development of T2D. While sex differences were not analyzed in the present study due a low sample size, future studies should investigate whether sex specific differences played a role in these findings. Further, given that our lean subjects were younger than our obese and obese T2D subjects, future studies should examine the impact of aging on these parameters.

In conclusion, the blunted leg blood flow response following 60 minutes of hyperinsulinemia in obese T2D individuals in the present study is not due to impaired production/activation of eNOS and ET-1 to insulin stimulation, but may be due to chronically higher basal levels of ET-1 content and expression and reduced peNOS/eNOS present at rest in T2D.

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Disclosures

The authors report no conflicts of interest.

Author Contributions

L.J.R, P.J.F, and J.P.T. conception and design of research; L.J.R., D.P.C., C.M.M., J.P.T. performed experiments; L.J.R., D.P.C., J.P. analyzed data; L.J.R. D.P.C. J.P. C.M.M. P.J.F and J.P.T. interpreted results of experiment; L.J.R prepared figures; L.J.R drafted manuscript; L.J.R. D.P.C. J.P. C.M.M. P.J.F and J.P.T. edited and revised manuscript; and L.J.R. D.P.C. J.P. C.M.M. P.J.F and J.P.T. approved final version of manuscript.

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586 **Figure Legend**

587 **Figure 1.** Serum glucose (panel A) and insulin (panel B) levels during the hyperinsulinemic-euglycemic
588 clamp in Lean individuals and obese individuals with type 2 diabetes (T2D). Percent change in femoral
589 artery blood flow from basal to 45 minutes of insulin stimulation in the Lean individuals and obese
590 individuals with T2D (panel C). Black circles represent the Lean individuals and the open circles
591 represent the obese T2D individuals. All samples were derived at the same time and processed in parallel.
592 *P<0.05 from Lean. Values are mean ± S.E.

593 **Figure 2.** Protein kinase B (AKT) (panel A), phosphorylation of AKT (panel pAKT) (B), endothelial
594 nitric oxide synthase (eNOS) (panel C), phosphorylation of eNOS (peNOS) (panel D), and endothelin-1
595 (ET-1) (panel E) peptide at basal and following 60 minutes of insulin stimulation in the Lean individuals
596 and obese individuals with type 2 diabetes (T2D). Representative blots for each protein measured (panel
597 F). Black bars represent the Lean individuals and open bars represent the obese T2D individuals. All
598 samples were derived at the same time and processed in parallel. Values are mean ± S.E.

599 **Figure 3.** NOS3 mRNA expression (panel A) and pre-pro ET-1 mRNA expression (panel B) at basal and
600 following 60 mins of insulin stimulation in the Lean individuals and obese individuals with type 2
601 diabetes (T2D). Black bars represent the Lean individuals and open bars represent the obese T2D
602 individuals. All samples were derived at the same time and processed in parallel. Values are mean ± S.E.

603 **Figure 4.** Protein kinase B (AKT) (panel A), phosphorylation of AKT (pAKT) (panel B), endothelial
604 nitric oxide synthase (eNOS) (panel C), phosphorylation of eNOS (peNOS) (panel D), and endothelin-
605 1(ET-1) (panel E) at rest in the Lean individuals, obese type 2 diabetes (T2D) individuals and obese
606 individuals without T2D. Representative blots for each protein measured (panel F). All samples were
607 derived at the same time and processed in parallel. Values are mean ± S.E.

Figure 1.

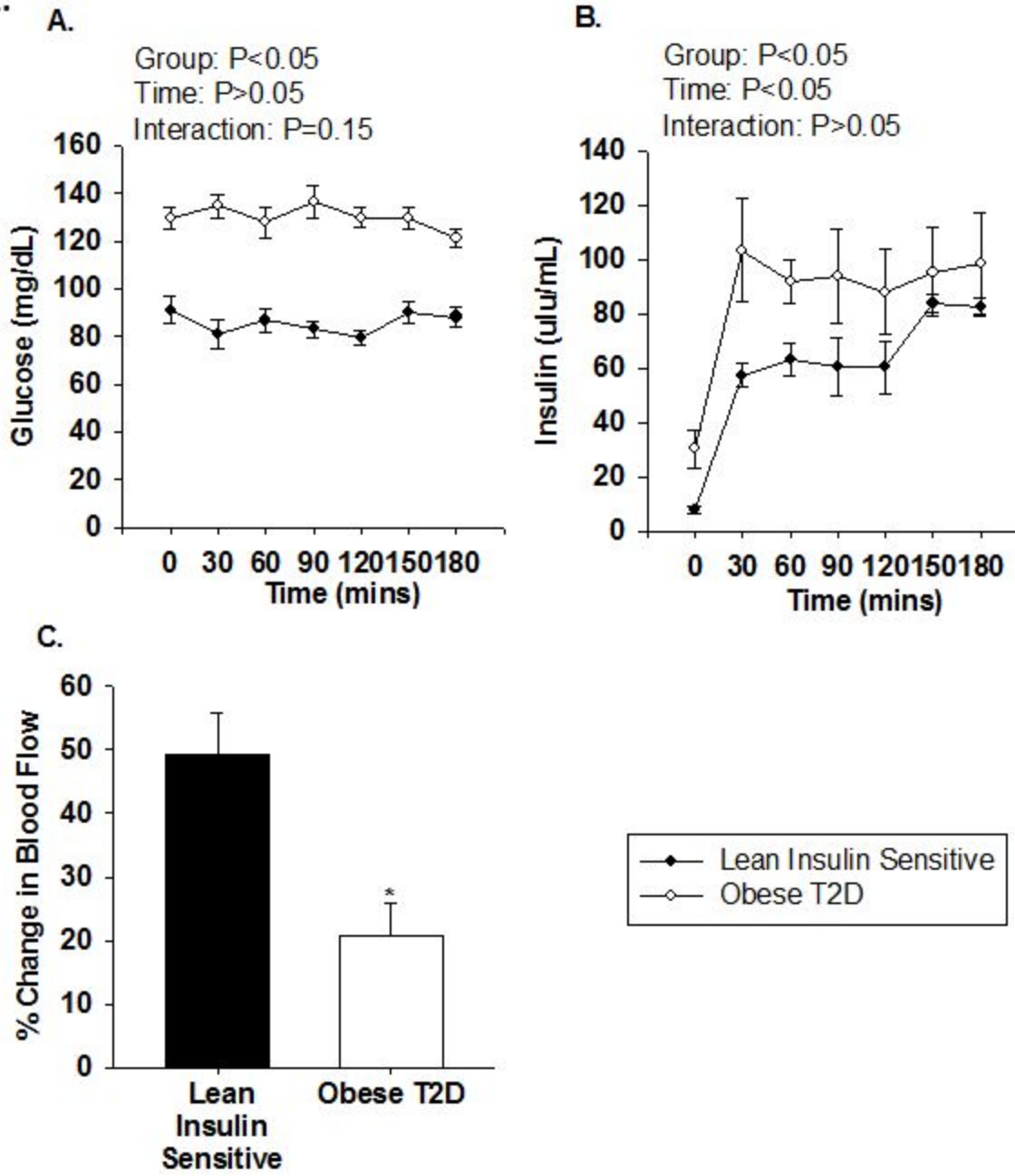

Figure 2.

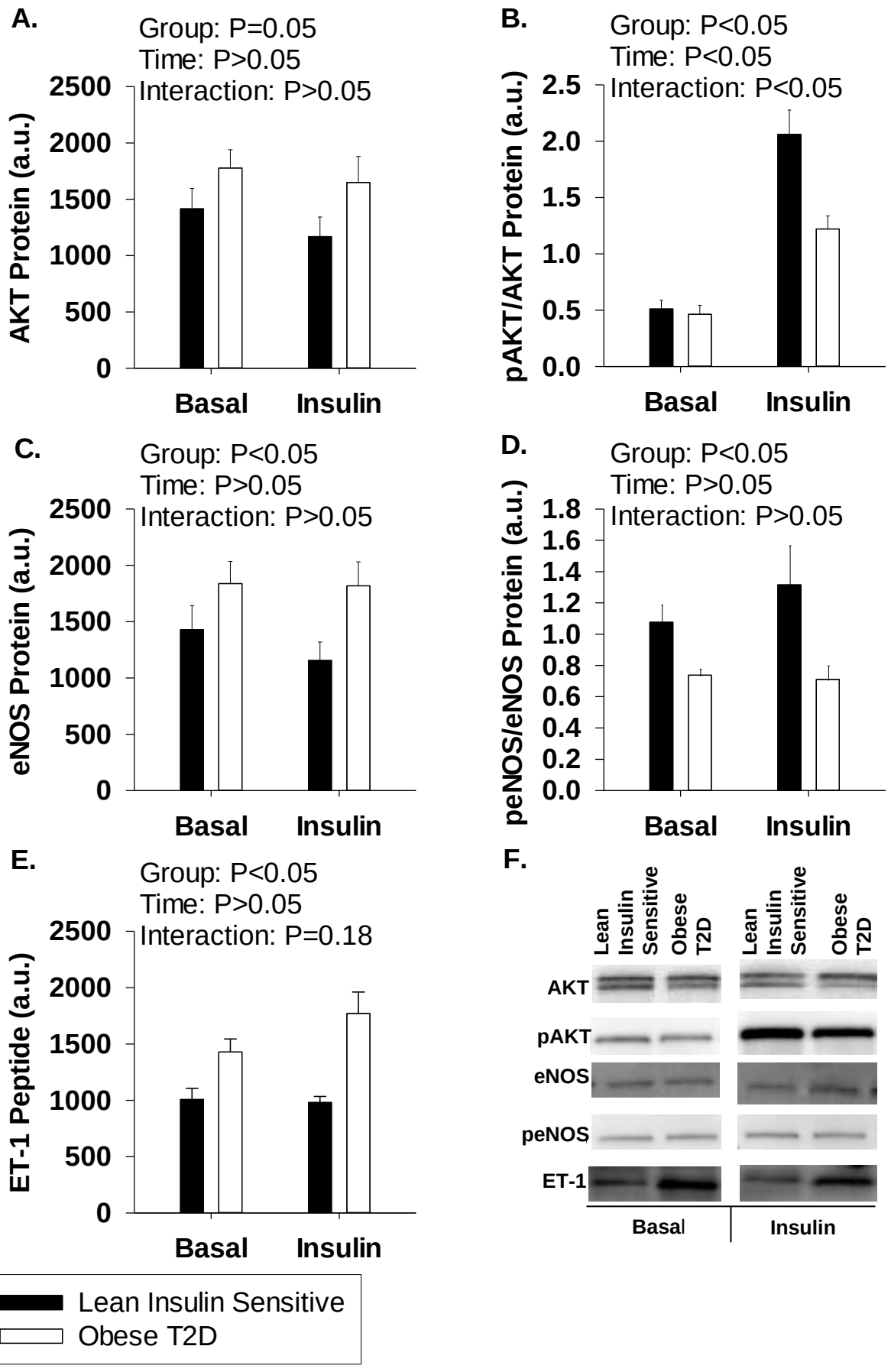
A.
Group: P=0.05
Time: P>0.05
Interaction: P>0.05
AKT Protein (a.u.)
2500
2000
1500
1000
500
0
Basal
Insulin
B.
Group: P<0.05
Time: P<0.05
Interaction: P<0.05
pAKT/AKT Protein (a.u.)
2.5
2.0
1.5
1.0
0.5
0.0
Basal
Insulin
C.
Group: P<0.05
Time: P>0.05
Interaction: P>0.05
eNOS Protein (a.u.)
2500
2000
1500
1000
500
0
Basal
Insulin
D.
Group: P<0.05
Time: P>0.05
Interaction: P>0.05
peNOS/eNOS Protein (a.u.)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Basal
Insulin
E.
Group: P<0.05
Time: P>0.05
Interaction: P=0.18
ET-1 Peptide (a.u.)
2500
2000
1500
1000
500
0
Basal
Insulin
F.
Lean Insulin Sensitive
Obese T2D
Lean Insulin Sensitive
Obese T2D
AKT
pAKT
eNOS
peNOS
ET-1
Basal
Insulin
Lean Insulin Sensitive
Obese T2D

Figure 3.

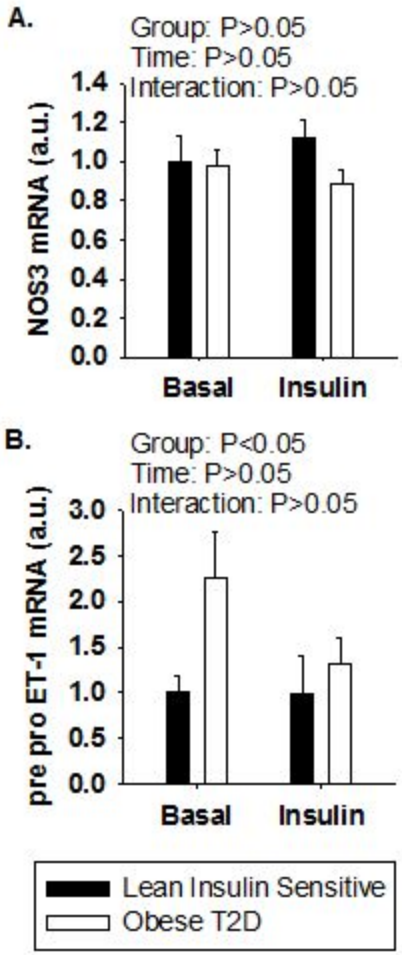

Figure 4.

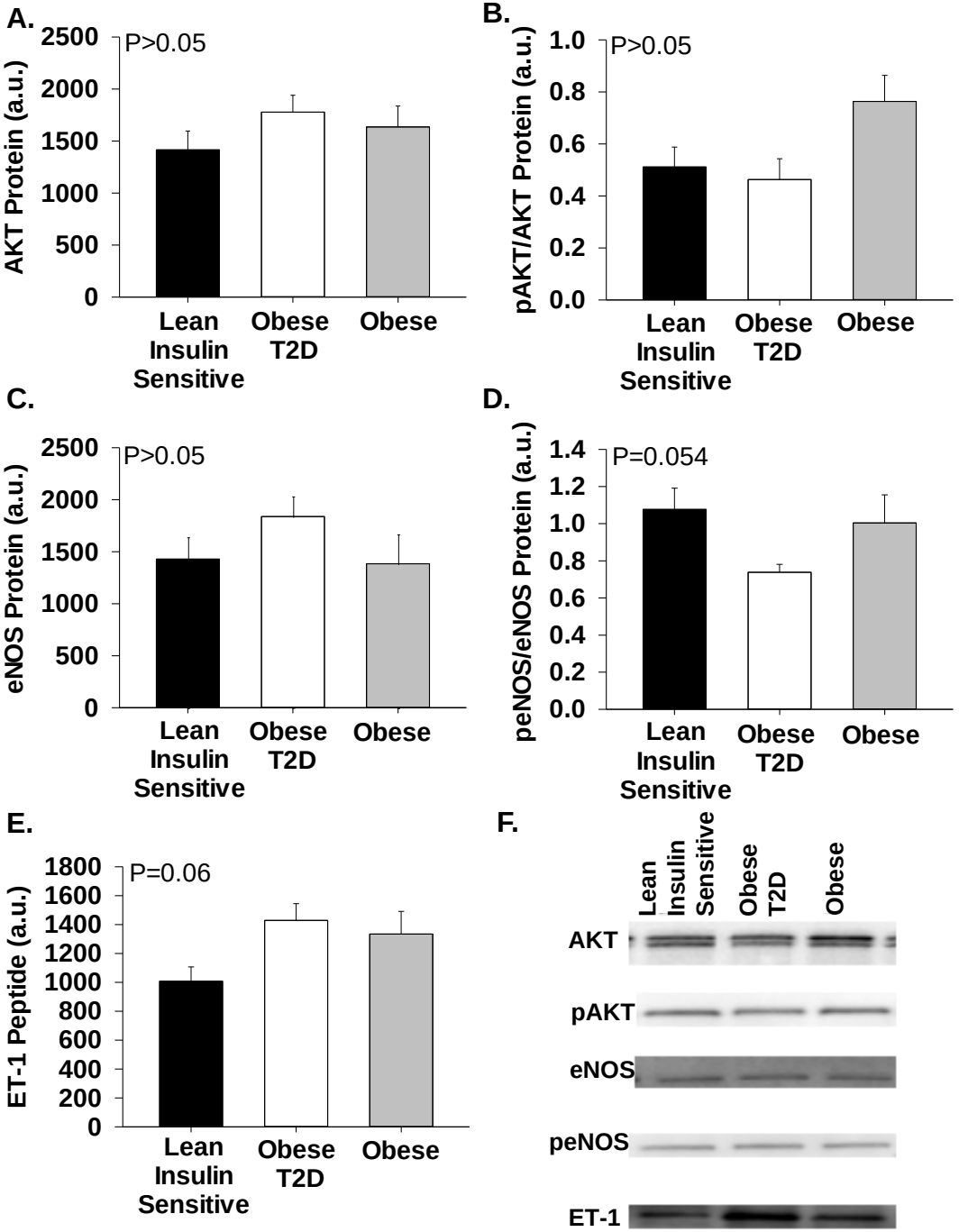

Table 1. Subject Characteristics

Baseline Subject Characteristics	Lean	Obese	Obese T2D
Age (yrs)	28±1.5	55±1.3*	55±1.7*
BMI (kg/m²)	25.3±1.1	34.8±1.4*	36.5±1.3*
Body Fat (%)	25.3±2.1	38.9±1.8*	41.3±2.2*
GDR (mg/kg LBM/min)	12.9±1.2		6.6±1.6*
HOMA IR	1.9±0.4	3.1±0.6	10.1±2.6*†
Glucose (mg/dL)	85±2.5	94±2.6	135±5.8*†
Insulin (μIu/mL)	8.3±1.8	13.2±2.5	30.3±6.6
Triglycerides (mg/dL)	72±5.6	149±27.0*	135±16.2*
Total Cholesterol (mg/dL)	167±12.7	215±11.1*	147±10.4†

*P<0.05 from Lean

†P<0.05 from Obese. Values are mean ± S.E.

Table 2. Subject Medications

	Lean	Obese	Obese T2D
Antidiabetic medications, N			
Biguanide	0	0	0
Sulfonylurea	0	0	2
Insulin	0	0	1
Cardiovascular medications, N			
β-Blocker	0	0	3
ACE Inhibitor	0	0	8
Calcium Channel Blocker	0	0	1
Diuretic	0	0	4
Statin	0	0	6
Fenofibrate	0	0	1
Others, N			
Levothyroxine	0	0	3

Values (N) are no. of subjects. T2D, type 2 diabetes