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Effects of Equivalent Sympathetic Activation during Hypoglycemia on Endothelial Function and Pro-Atherothrombotic Balance in Healthy Individuals and Obese Standard Treated Type 2 Diabetes

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Abstract

Objective—Recent studies in type 2 diabetes have reported an association between hypoglycemia and severe cardiovascular adverse events, which are relatively increased in standard versus intensively treated individuals. The aim of this study was to determine the effects of equivalent sympathetic nervous system (SNS) activity during moderate hypoglycemia on in-vivo endothelial function, pro-inflammatory, pro-atherothrombotic, and pro-coagulant responses in healthy and standard treated type 2 diabetes individuals.

Research design and methods—Eleven type 2 diabetes and 16 healthy individuals participated in single 2 day studies. Day 1 involved a 2 hr hyperinsulinemic/euglycemic clamp and day 2, a 2 hr hyperinsulinemic/hypoglycemic clamp of 3.2 ± 1 mmol/L in type 2 diabetes and (2.9 ± 0.1 mmol/L) in healthy individuals.

Results—ICAM-1, VCAM-1, P-selectin, PAI-1, VEGF and endothelin-1 (ET-1) fell during hyperinsulinemic euglycemia but increased during hypoglycemia in type 2 diabetes and healthy individuals. Epinephrine and norepinephrine levels were equivalent during hypoglycemia in type 2 DM and healthy individuals. However, despite similar SNS drive but milder and hypoglycemia there were greater ICAM-1, VCAM-1, PAI-1, VEGF and ET-1 responses in the type 2 diabetes group. Endogenous and exogenous nitric oxide mediated arterial vasodilation were also impaired only during hypoglycemia in type 2 diabetes.

Conclusion—We conclude that, milder hypoglycemia but equivalent SNS activation results in more diffuse endothelial dysfunction and a greater pro-inflammatory, pro-atherothrombotic and pro-coagulant state in standard treated type 2 diabetes as compared to healthy individuals.

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Keywords

hypoglycemia; endothelial function; type 2 diabetes; sympathetic nervous system; atherothrombosis

1. INTRODUCTION

Despite the recent decline of cardiovascular deaths in the general population, patients with diabetes have not followed the same trend [1]. Type 2 diabetes patients have early development of endothelial dysfunction, platelet hyperactivity, aggressive atherosclerosis, increased inflammation and impaired fibrinolysis resulting in greater atherothrombotic events [2]. Recently the Edinburg Type 2 Diabetes Study has reported that hypoglycemia is associated with increased levels of inflammatory markers and is an independent risk factor for macro-vascular events [3]. Unfortunately, severe hypoglycemia occurs in both intensive and standard treated type 2 diabetes mellitus patients [4–6]. In fact, individuals in the standard treatment arms of the above studies appeared to have a greater risk of severe cardiovascular events and death associated with severe hypoglycemia as compared to the intensively treated groups. Despite the available information from large type 2 diabetes epidemiologic and glucose control and complications studies linking hypoglycemia with severe cardiovascular adverse events [4–6], there are scarce physiologic data available in type 2 diabetes humans examining the pathophysiologic effects of hypoglycemia on endothelial function, pro-inflammatory and atherothrombotic biomarker responses. Additionally, there are no data comparing the acute pro-inflammatory and pro-atherothrombotic responses during hypoglycemia in type 2 diabetes and non-diabetic individuals. Finally, there are no data examining the comparable effects of SNS activation during hypoglycemia in type 2 diabetes and healthy individuals. This appears relevant as SNS activation (epinephrine, norepinephrine or direct sympathetic nervous system drive) is considered to be a principal putative mechanism for the pro-inflammatory and pro-atherothrombotic responses occurring during hypoglycemia in non-diabetic humans [7–11]. Thus, this present study has tested the hypothesis that due to a background of increased inflammation and endothelial dysfunction, moderate hypoglycemia with equivalent SNS drive would produce greater reductions in fibrinolytic balance ($\uparrow$ PAI-1) and increased pro-inflammatory and pro-atherothrombotic responses in obese type 2 diabetes individuals as compared to healthy individuals.

2. RESEARCH DESIGN AND METHODS

2.1 Study Participants

Eleven type 2 diabetes (2M/9F) (45 ± 4 yrs, BMI 38 ± 3 kg/m², HbA1c $8 \pm 1\%$, 64 ± 10.9 mmol/mol) and 16 healthy (11M/5F) (36 ± 3 yrs, BMI 27 ± 1 kg/m², HbA1c $5 \pm 0.3\%$, 31 ± 3.3 mmol/mol) individuals were studied (Table: 1). Each participated in a single 2 day study. All had normal hematologic, renal and liver function tests. Type 2 diabetes individuals had a mean duration of 6 yrs diabetes and were treated with metformin (n=11), sulfonylureas (n=3), DPP-4 inhibitors (n=2), exenatide (n=1) or rapid (n=2), intermediate (n=2), or long-acting insulin (n=3). None of the subjects had tissue complications of diabetes. None of the

participants smoked, received anticoagulants, clopidogrel, or thiazolidinediones. Individuals over age 40 were screened for silent ischemia with a standard Bruce protocol treadmill stress test [12]. None of the type 2 diabetes participants had suffered any hypoglycemia in the preceding week before the study. All gave written informed consent. Some of the data from non-diabetic individuals have been included in a previous report [13]. Studies were approved by the Vanderbilt University and University of Maryland Human Subjects Institutional Review Boards.

2.2 Experimental Design

Participants were instructed to avoid any exercise and consume their usual weight maintaining diet for 3 days before each experiment. Participants were also asked not to use aspirin, NSAIDs, COX-2 inhibitors, or phosphodiesterase 5 inhibitors three days prior to the study. Also metformin, sulfonylureas, DPP-4 inhibitors, exenatide and any long-acting insulin were stopped 3 days prior to admission. For the 3 days prior to admission all type 2 diabetes subjects reported glucose values 4 times per day and received pre-prandial rapid acting insulin to maintain pre-prandial glucose levels of 5–8 mmol/L and avoid hypoglycemia. Participants were admitted to the general clinical research center (GCRC) during the evening prior to the study. Upon admission, two intravenous (IV) lines were placed in the hand and arm of the individual under local anesthesia (0.1 ml of 1% lidocaine, subdermally). One line was inserted in a retrograde fashion in a vein on the back of the hand and was used to draw blood samples during the study days. This hand was placed in a heated box (55–60°C) during the study so that arterialized blood could be obtained [14]. The other cannula was placed in the ipsilateral or contralateral arm for infusions. A standard evening meal and snack was consumed and a variable dose IV insulin infusion was used to maintain equivalent overnight glucose control of 5–8 mmol/L in type 2 diabetes individuals before euglycemic or hypoglycemic clamps.

Day1- Euglycemia—Following an overnight 10 hr fast there was a 120 min basal period followed by a 120 min hyperinsulinemic-euglycemic clamp study. At time 120 min, a primed constant IV infusion of insulin 12 pmol/kg/min in type 2 diabetes and 9 pmol/kg/min in healthy controls [15] was started and continued until 240 min (Figure 1). An increased dose of insulin was used in the type 2 diabetes individuals to control for the elevated levels of insulin needed to create and sustain hypoglycemia during the day 2 studies. During this clamp period, plasma glucose was measured every five min and a 20% dextrose infusion was adjusted so that plasma glucose levels were held constant at 5 ± 0.1 mmol/L [16]. Potassium chloride (5 mmol/hr) was infused to reduce insulin-induced hypokalemia. At the end of the study all participants received a standard lunch and evening meal and then underwent a 10 hr overnight fast. Glycemic control 5–8 mmol/L was maintained throughout the remainder of the day and night in the type 2 diabetes individuals by a variable dose IV insulin infusion.

Day 2- Hypoglycemia—Similar to day 1 there was an initial 120 min basal equilibration period. At time 120 min a primed constant IV infusion of regular insulin (identical to day 1) was started and continued until 240 min. The rate of fall of glucose was controlled (≈ 0.06 – 0.08 mmol/L/min) and the glucose nadir of 3.2 mmol/L in type 2 diabetes and 2.9 mmol/L in healthy controls were achieved at time 150 min and maintained until time 240 min using a

modification of the glucose clamp technique [16, 17]. Potassium chloride (5 mmol/hr) was infused during the clamp.

2.3 Endothelial Function

Measurements of endothelial function were conducted at baseline and during the final 30 minutes of each glucose clamp. Flow mediated dilation (FMD) of the brachial artery was measured using 2D Doppler ultrasound during reactive hyperemia and nitroglycerin administration (Philips iE33 ultrasound system, Philips Medical Systems, Bothell WA.) as previously described [13]. Endothelium dependent NO mediated vasodilation was obtained by inflating the blood pressure cuff around the proximal forearm to a pressure of 50 mmHg greater than the patient's systolic blood pressure for 5 minutes [18]. Brachial artery diameter measurements were taken at time points 30, 60, 90 and 120 seconds after cuff deflation. Then after a 15–20 minute rest period, subjects received 0.4 mg sublingual nitroglycerin to determine exogenous nitric oxide mediated endothelial vasodilation. Additional scans were performed as above with vessel diameter measurements obtained at 1, 2, 3 and 4 minutes. The coefficient of variation (CV) at baseline and end of glucose clamps in healthy and type 2 diabetes groups for FMD measurements was <1%.

2.4 Cardiovascular Measurements

Heart rate and systolic, diastolic and mean arterial blood pressure were measured noninvasively by a Dinamap vitals monitor (Critikon, Tampa, FL) every 10 min throughout each 2 hr clamp study.

2.5 Statistical Analysis

Data are expressed as mean $\pm$ SE. Baseline values consisted of the mean of two time points drawn before starting the clamp procedure. End of clamp values were measured during the final 30 min of euglycemic or hypoglycemic studies. Response during a glucose clamp (either euglycemic or hypoglycemic) consisted of the baseline value subtracted from the end of clamp value. After testing for equal variances (F test), paired 2 tailed t-tests were used to determine if end of clamp values increased or decreased from baseline and whether end of clamp responses from baseline were different during day 1 euglycemic as compared to day 2 hypoglycemic clamps for either healthy or type 2 diabetes individuals. If variances were not equal then non-parametric analysis (Wilcoxon matched pairs signed rank test) were used for the above comparison. (Graph Pad Software, Inc., San Diego, CA). Between group comparisons of baseline and end of clamp values between healthy and type 2 diabetes individuals utilized standard parametric one way analysis of variance if data passed Bartlett's test for equal variance or Kruskal-Wallis test if there were unequal variances in any parameter. Once an overall group difference was identified by ANOVA, within group analysis using a multiple comparison test (eg. Tukey's test for parametrics) or Dunn's multiple comparison test (non-parametric) were used to identify post hoc differences between individual groups. Responses during hypoglycemia are relatively large and consistent. Therefore, a power calculation was based on the expected (and known) responses of PAI-1 (which was used as the primary variable) that indicated the mean value during hypoglycemia (27 ng/ml) compared to the value during euglycemia (16 ng/ml) with a common standard deviation of 13 ng/ml and 9 ng/ml respectively set at an alpha error level

of 5% and a beta error level of 80% using a paired t-test with a 0.05 two sided significance level indicated that a sample size of 11 would be required to find differences between groups (nQuery Advisor version 3.0, Statistical Solutions, Saugus, MA).

2.6 Analytical Methods

The collection of blood samples has been described elsewhere [19]. Plasma glucose concentrations were measured in triplicate using the glucose oxidase method with a glucose analyzer (Beckman, Fullerton, CA). Insulin was measured as previously described with an interassay coefficient of variation (CV) of 9% [20]. Catecholamines were determined by HPLC with an interassay of 12% for epinephrine and 8% for norepinephrine [21]. Cortisol was assayed using the clinical assays gamma coat RIA kit with an interassay CV of 6%. NEFA were measured using the WAKO kit with an interassay CV of 7% [22]. Glucagon was measured according to a modification of the method of Aguilar-Parada et al [23] with an interassay CV of 12%.

Vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, tumor necrosis factor-alpha (TNF- α) and vascular endothelial growth factor (VEGF) were assayed using LINCO Research Kits (St. Charles, Missouri) with an interassay CV of 8.5%, 9.7%, 13.4%, 9.98% and 10% respectively. P-selectin was measured by Meso Scale Discovery (Gaithersburg, MD) with an interassay CV of 9.9%. Plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (tPA) were determined by TintElize® Platinum Kits (St. Charles, MO) with an interassay CV of 3.3% and 6.5%, respectively. Endothelin-1 (ET-1) was measured by R&D System's Quantikine ELISA Kit (Minneapolis, MN) with an interassay CV of 5.9%.

3. RESULTS

3.1 Glucose, Insulin and Glucose Infusion Rates

Plasma glucose was maintained at 5 ± 0.1 mmol/L during the euglycemic clamps. During the hypoglycemic clamp studies, plasma glucose reached a steady state by 150 min and was maintained at a stable plateau of 3.2 ± 0.1 mmol/L in the type 2 diabetes group and 2.9 ± 0.1 mmol/L in healthy controls (Figure:1). Insulin levels were 1167 ± 50 pmol/L in the type 2 diabetes individuals and 812 ± 56 pmol/L in healthy individuals respectively (Figure:1). Glucose infusion rates to maintain euglycemia on day 1 were 3.5 ± 0.7 and 7.0 ± 0.3 mg/kg/min for individuals with type 2 diabetes and healthy individuals respectively. Glucose infusion rates during hypoglycemia were 0.6 ± 0.1 and 1.0 ± 0.3 mg/kg/min for individuals with type 2 diabetes and healthy respectively.

3.2 Autonomic Nervous System Counterregulatory Hormones

Baseline levels of autonomic nervous system hormones were similar at the start of the euglycemic and hypoglycemic clamps in both healthy and type 2 diabetes groups (Table:2). Epinephrine levels remained similar to baseline during euglycemia (277 ± 91 pmol/L and 189 ± 20 pmol/L) in individuals with type 2 diabetes and healthy individuals, respectively (Table:2). Epinephrine levels were equivalently increased compared to euglycemia ($p < 0.001$) during the final 30 min of the hypoglycemic clamps 4049 ± 970 pmol/L and 4251

± 568 pmol/L in individuals with type 2 diabetes and healthy respectively. Norepinephrine levels were also equivalently higher ($p < 0.001$) during the final 30 min of hypoglycemia (1907 ± 155 and 1957 ± 130 pmol/L) as compared to euglycemia (1003 ± 59 and 1159 ± 75 pmol/L) in the type 2 diabetes individuals and healthy individuals respectively (Table:2).

3.3 Neuroendocrine Counterregulatory Hormones

Baseline levels of neuroendocrine counterregulatory hormones were similar at the start of the euglycemic and hypoglycemic clamps in both healthy and type 2 diabetes groups (Table: 2). Cortisol and glucagon levels were significantly increased ($p < 0.001$) during hypoglycemia in both healthy and type 2 diabetes groups as compared to euglycemia (Table:2). Cortisol and glucagon levels were also relatively increased ($p < 0.02$) during the final 30 min of hypoglycemia in individuals with type 2 diabetes as compared to healthy individuals (Table: 2). Growth hormone and pancreatic polypeptide levels were similar during hypoglycemia in both groups.

3.4 Intermediary Metabolism

NEFA baseline levels were similar at the start of glucose the clamps. Blood NEFA levels fell from baseline ($p < 0.001$) during both hypoglycemic and euglycemic clamps (Table:2). However, end of clamp NEFA levels were higher ($p < 0.01$) during hypoglycemia in individuals with type 2 diabetes as compared to euglycemia and hypoglycemia in healthy individuals (Table:2).

3.5 Vascular Adhesion Molecules ICAM-1, VCAM-1 and E-selectin

Baseline values of VCAM-1 were lower in the type 2 diabetes group (Table:3) at the start of euglycemic and hypoglycemic clamps as compared to healthy individuals ($p < 0.01$). ICAM-1 and VCAM-1 fell ($p = 0.03-0.003$) during both series of hyperinsulinemic euglycemic clamps in type 2 diabetes and healthy individuals respectively (Figure:2). ICAM-1 and VCAM-1 levels increased from baseline during both series of hypoglycemic studies ($p = 0.01-0.009$). However, increases in ICAM-1 and VCAM-1 were greater ($p = 0.01-0.005$) during hypoglycemia in individuals with type 2 diabetes as compared to healthy individuals (Figure:2). Baseline values of E-selectin were similar and fell similarly during both series of euglycemic clamps (-2.5 ± 1 , -2 ± 1 ng/mL in type 2 diabetes and healthy individuals respectively $p < 0.04$). E-selectin increased similarly during hypoglycemia in type 2 diabetes and healthy groups respectively (2.8 ± 1 ; 1.7 ± 1 ng/mL, $p < 0.005$).

3.6 P-selectin, PAI-1 and tPA

Baseline values for P-selectin and PAI-1 at the start of the euglycemic and hypoglycemic clamps are shown in Table 3. Plasma P-selectin levels fell from baseline during euglycemic studies ($p < 0.002$) in healthy individuals and individuals with type 2 diabetes and increased similarly during hypoglycemia in both individuals with type 2 diabetes and healthy individuals ($p < 0.001$) (Figure:2). PAI-1 levels fell by a greater extent during euglycemia in type 2 diabetes as compared to healthy ($p < 0.001$) (Figure: 2). PAI-1 remained similar to baseline during hypoglycemia in individuals with type 2 diabetes (Figure: 2), which represented a severe blunting and thus a significant increase ($p < 0.005$) compared to the fall

during euglycemic studies. The magnitude of the difference of PAI-1 responses between euglycemia and hypoglycemia was greater ($p<0.03$) in type 2 diabetes as compared to healthy individuals. PAI-1 responses in healthy individuals were increased from baseline during hypoglycemia ($p<0.04$) and were also elevated relative to euglycemia ($p<0.04$) (Figure: 2). Basal blood levels of tPA were reduced ($p=0.01$) in individuals with type 2 diabetes as compared to healthy individuals but remained unchanged compared to baseline in both groups during euglycemic and hypoglycemic clamps.

3.7 VEGF, ET-1, and TNF- α

Baseline values of VEGF were higher ($p<0.02$) in type 2 diabetes as compared to healthy individuals. VEGF decreased by a greater amount ($p=0.02$) during euglycemia and increased by a greater extent ($p<0.004$) during hypoglycemia in type 2 diabetes relative to healthy individuals (Figure:3). Baseline values of ET-1 were similar at the start of euglycemic and hypoglycemic studies. ET-1 fell similarly during euglycemic studies in both type 2 diabetes and healthy individuals. ET-1 levels also fell during hypoglycemia in healthy. However, ET-1 responses were increased ($p<0.01$) in individuals with type 2 diabetes during hypoglycemia relative to baseline, euglycemia and healthy individuals. Baseline values of TNF- α were similar at the start of the euglycemic and hypoglycemic clamps in healthy and type 2 diabetes individuals (Table 3). TNF- α levels fell by a similar amount during euglycemia in type 2 diabetes ($0.08\pm0.3\text{pg/ml}$) as compared to healthy individuals ($0.04\pm0.2\text{pg/ml}$). TNF- α was increased similarly ($p<0.04$) during hypoglycemia in individuals with type 2 diabetes ($0.2\pm0.2\text{pg/ml}$) and healthy individuals ($0.3\pm0.2\text{pg/ml}$).

3.8 Endothelial Function

Basal brachial artery diameters were similar at the start of the euglycemic and hypoglycemic studies in both type 2 and healthy individuals ($0.44\pm0.02\text{mm}$). Brachial artery diameters were also similar to baseline during each euglycemic and hypoglycemic clamp ($0.42\text{--}0.44\pm0.02\text{mm}$). Flow mediated dilation (endothelial-dependent vasodilation) was similar during euglycemia in individuals with type 2 diabetes and healthy individuals. Flow mediated endothelial-dependent vasodilation was reduced from baseline in both groups, but by a greater extent during hypoglycemia in type 2 diabetes as compared to healthy individuals (Figure: 3). Nitroglycerin mediated exogenous NO dependent vasodilation remained similar to baseline during euglycemic studies in both type 2 diabetes and healthy individuals. During hypoglycemia nitroglycerin mediated exogenous NO dependent vasodilation was reduced in type 2 diabetes but not in healthy individuals (Figure: 3). The magnitude of change of flow mediated endothelial dependent vasodilation did not correlate with the magnitude of change of any of the above measured biomarkers.

3.9 Cardiovascular Parameters

Heart rate, systolic, diastolic and mean arterial blood pressure responses are reported in Table: 4.

4. DISCUSSION

This present study has demonstrated that in the presence of milder hypoglycemia, equivalent sympathetic nervous system drive can produce increased pro-inflammatory, pro-atherothrombotic (ICAM-1, VCAM-1, PAI-1, P-selectin, VEGF, Endothelin-1) and decreased arterial endothelial responses in obese, standard controlled type 2 diabetes as compared to healthy non-obese individuals.

Counterregulatory responses to hypoglycemia differ according to glycemic control in type 2 diabetes individuals [24], [25]. Tighter glycemic control reduces sympathetic nervous system and neuroendocrine responses, whereas less strict glycemic control results in higher glycemic thresholds for hormone release and increased counterregulatory responses during hypoglycemia [25], [26].

Recent large glucose control and complications trials in type 2 diabetes [4–6] have reported relatively greater associations of severe cardiovascular adverse events following hypoglycemia in standard as compared to intensively treated individuals. The physiologic mechanisms for these findings have not been identified. A plausible explanation is that due to reduced antecedent hypoglycemia, there are increased SNS responses in the standard control groups that may be responsible for the greater incidence of severe cardiac events. Therefore, the purpose of this report was to determine the effects of equivalent sympathetic activation during hypoglycemia on pro-inflammatory and pro-atherothrombotic responses in a group of type 2 diabetes individuals and comparator group of non-diabetic individuals. Hypoglycemia has been reported to activate pro-inflammatory and pro-atherothrombotic responses in healthy and type 1 diabetes individuals for over 30 years [27–30]. This present study is the first to our knowledge that confirms mild/moderate hypoglycemia in type 2 diabetes can also acutely activate a similar wide spectrum of pro-inflammatory and pro-atherothrombotic responses. ICAM-1 and VCAM-1 are vascular adhesion molecules involved in atherosclerotic development [31, 32]. Both of these biomarkers were suppressed similarly during hyperinsulinemic euglycemia in type 2 diabetes and healthy individuals. However, both increased during hypoglycemia, but by a greater extent in type 2 diabetes compared to healthy individuals.

E-Selectin, a cell adhesion molecule, P-selectin, a marker of platelet aggregation [33], and TNF- α , a systemic cytokine inflammatory molecule [34], all fell similarly in both groups during the euglycemic studies. E-selectin, P-selectin, and TNF- α increased similarly during hypoglycemia, which represents a relatively greater response to the milder hypoglycemia in type 2 diabetes as compared to healthy individuals. Many studies have established that fibrinolytic dysfunction (elevated PAI-1 relative to tPA) is an important mediator for the increased risk of coronary thrombotic disease in individuals with increased insulin resistance and type 2 diabetes [35]. There are only a few studies reporting the effects of hypoglycemia on fibrinolytic/coagulation mechanisms in healthy and type 1 diabetes individuals [28, 29, 36], but no data reporting these effects during hypoglycemia in type 2 diabetic humans. PAI-1, a critical inhibitor of fibrinolysis, fell during euglycemia in healthy individuals, and by a greater extent in type 2 diabetes. During hypoglycemia PAI-1 levels increased in healthy individuals and the reduction in PAI-1 that occurred during euglycemia was

dramatically suppressed in the type 2 diabetes group. This resulted in a relative change of PAI-1 during euglycemia and hypoglycemia that was far greater in the type 2 diabetes group. Plasma levels of tPA, the primary mediator of fibrinolysis remained similar to baseline during euglycemic and hypoglycemic studies in both type 2 diabetes and healthy individuals. However, tPA levels were several fold lower in the type 2 diabetes group with the result that fibrinolytic balance was markedly reduced during hypoglycemia in type 2 diabetes as compared to the healthy group.

It is of note that despite comparable SNS counterregulatory drive there were greater NEFA levels during the type 2 diabetes hypoglycemic studies. This is no doubt due to the greater adipose tissue mass in type 2 diabetes individuals. Previous work has demonstrated that NEFA can have pro-inflammatory and platelet hyperaggregability effects [37–39]. Therefore, it is possible that the elevated NEFA levels (secondary to the SNS drive) during hypoglycemia in the type 2 diabetes group could also have contributed mechanistically to the present findings.

Endothelin-1 (ET-1) is a potent vasoconstrictor that has been reported to increase during hypoglycemia in type 1 diabetes [36, 40, 41]. There are no available data reporting whether ET-1 also increases during hypoglycemia in type 2 diabetes. ET-1 decreased by similar amounts during euglycemic studies in healthy and type 2 diabetes individuals. ET-1 remained suppressed during hypoglycemia in healthy individuals but increased significantly in individuals with type 2 diabetes.

Our findings are consistent with previous work demonstrating increased activity of vascular ET-1 during non hypoglycemic conditions in type 2 diabetes [42]. ET-1 is implicated in the pathogenesis of insulin resistance and has also been demonstrated to be a potent mediator of endothelial dysfunction in obese and type 2 diabetes individuals [27, 36]. It is therefore likely that ET-1 contributed to the greater reduced endogenous and exogenous NO mediated endothelial function occurring in the type 2 diabetes individuals during hypoglycemia. The regulation of ET-1 release during stress is incompletely understood. There are competing reports of insulin either stimulating or having no effect on ET-1 release [43, 44]. We are not aware of any data addressing whether SNS activity can directly increase ET-1 release in vivo. Similarly it is unknown whether hypoglycemia per se could activate vascular ET-1 release. VEGF has also been implicated in the pathogenesis of insulin resistance [2]. VEGF levels are known to be considerably higher in type 2 diabetes as compared to healthy individuals and are involved in the pathogenesis of diabetic renal and microvascular complications [2]. Hyperinsulinemia had a greater effect in lowering VEGF levels during euglycemia in type 2 diabetes individuals. However, VEGF responses were amplified during hypoglycemia in type 2 diabetes as compared to healthy individuals.

Taken together, the above effects on ICAM-1, VCAM-1, P-selectin, VEGF, Endothelin-1, and fibrinolytic balance indicate that, acutely milder hypoglycemia but equivalent SNS drive (as demonstrated by equivalent adrenomedullary epinephrine and sympathoneural norepinephrine responses) can create a greater atherothrombotic and pro-coagulant state in type 2 diabetes compared to healthy individuals.

Endothelial function was measured non-invasively by testing both endogenous (endothelium-dependent) and exogenous (endothelium independent) NO mediated vasodilatory mechanisms. Brachial artery diameters were similar during the endothelial function tests in both type 2 diabetes and non-diabetic, healthy groups, both at baseline and during both sets of hyperinsulinemic euglycemic and hypoglycemic clamps. This provides a comparable and stable platform to compare acute endothelial function responses during the experimental physiologic stress. During hyperinsulinemic euglycemia, endothelial function was similar between the type 2 diabetic group and healthy individuals. It should be noted that insulin levels were 35% increased in the type 2 diabetes group and thus endothelial function may be considered relatively reduced compared to healthy controls. However, during hypoglycemia, endothelial function responses were clearly and substantially reduced by a greater extent in the type 2 diabetes as compared to the healthy group. Both endogenous and exogenous NO mediated vasodilation were reduced in the type 2 diabetes individuals, whereas only endogenous mediated vasodilation was reduced in healthy individuals. Thus moderate hypoglycemia of only 3.2 mmol/L in type 2 diabetes group was able to impair both endogenous vascular smooth muscle NO and prevent exogenous NO donors from activating protective arterial vasodilatory mechanisms [7].

We can exclude hyperinsulinemia as a causative mechanism for any of the pro-inflammatory, pro-atherothrombotic or reduced endothelial function biomarker responses occurring during hypoglycemia. Insulin levels were equated within each group (type 2 diabetes or healthy individuals) during both series of euglycemic and hypoglycemic studies. Pro-inflammatory and pro-atherothrombotic responses were suppressed while endothelial function was maintained during hyperinsulinemic euglycemia. These findings add to data that insulin, acutely, has anti-inflammatory actions in healthy, type 1 and type 2 diabetes individuals [28, 45, 46]. We should indicate that the higher insulin levels during hypoglycemia in type 2 diabetes individuals would have been predicted to reduce acute pro-inflammatory responses and thus would have reduced the experimental signal. Similarly, glycemia, glucagon and cortisol levels were also increased during hypoglycemia studies in type 2 diabetes, all of which would have been predicted to reduce pro-inflammatory responses [47, 48]. Therefore, we believe that the present finding of the increased pro-atherothrombotic and pro-inflammatory results during the type 2 diabetes hypoglycemia are conservative and could in fact be higher in clinical practice, where insulin levels causing hypoglycemia are much lower.

Several previous studies investigating the effects of adrenergic stimulation or blockade have reported important pro-inflammatory and pro-coagulant effects of circulating catecholamines or direct SNS drive [7–11]. These physiologic effects occur via several adrenergic receptor subtypes and include platelet activation ($\alpha_{1/2}$, $\beta_{1/2}$) increased coagulation (β_2), decreased fibrinolytic (β_2) and reduced endothelial function (α_1, α_2) [7–11]. All of the above pro-inflammatory vascular effects also occur during hypoglycemia. It thus allows the plausible hypothesis that the increased SNS drive occurring during hypoglycemia contributes to the observed pro-inflammatory responses. Complete blockade of SNS activity during hypoglycemia is challenging and is potentially hazardous particularly so in type 2 diabetes. Thus to begin to address this important clinical question, the present study compared the effects of an equivalent increase in SNS drive during acute

hypoglycemia in type 2 diabetes and healthy individuals. In order to ensure comparable SNS responses in the two groups, type 2 diabetes individuals with intact and robust counterregulatory responses (shorter duration of disease, rarely experienced hypoglycemic episodes, moderate glycemic control) were studied. In non-diabetic individuals the hypoglycemic threshold for counterregulatory hormone release occurs around ~70 mg/dL and hormone levels double for each subsequent 10 mg/dL decrements of plasma glucose [49]. As counterregulatory hormone responses occur at higher glucose levels in standard treated type 2 diabetes, a hypoglycemic difference of 6 mg/dL between the two study groups was needed to create equivalent SNS activation in type 2 diabetes and healthy individuals. However, the present results, demonstrate for the first time that similar to counterregulatory hormone release, there appears to be greater “sensitivity” of several pro-inflammatory and pro-atherothrombotic responses to hypoglycemia in standard controlled, obese type 2 DM as compared to healthy individuals.

Our study design requires further comment. We investigated a group of relatively young, shorter disease duration type 2 diabetes individuals with moderate glucose control, which could provide robust neuroendocrine and SNS counterregulatory responses. Our type 2 diabetes group was obese (BMI 38 kg/m²) and insulin resistant, which is reflective of many type 2 diabetes individuals living in the USA. In order to achieve even moderate hypoglycemia in our type 2 diabetes group we had to use 35% higher insulin levels as compared to our healthy controls (but similar to insulin levels used in our previous hypoglycemia studies in type 2 diabetes [25]. Insulin has been demonstrated to have antiinflammatory effects [45, 50]. Thus, we believe it was important to have euglycemic hyperinsulinemic studies to control for the independent anti-inflammatory effects of insulin in both, healthy and type 2 diabetes groups. Hypoglycemia results in increased insulin resistance, which is dependent upon the robustness of the counterregulatory response, and can last 2–3 days [51]. Therefore, we performed the antecedent hyperinsulinemic euglycemia control studies on day 1 as numerous studies have demonstrated that this approach does not affect neuroendocrine and SNS counterregulatory responses during next day hypoglycemia [13, 49, 52]. The hypoglycemic stimulus in the type 2 diabetes group was relatively moderate at 3.2 mmol/L. However, due to the robust counterregulatory response in type 2 diabetes individuals, ~45% of participants required no glucose infusion during the hypoglycemic clamps to maintain and defend a plasma glucose of 3.2 mmol/L against pharmacologic insulinemia. This underscores the importance of an intact counterregulatory response in protecting against a falling plasma glucose in individuals with type 2 diabetes but also precludes us from commenting whether deeper hypoglycemia would have produced even greater changes in vascular biologic markers.

In summary, this study demonstrates that relatively mild hypoglycemia in obese standard treated type 2 diabetes individuals produces acute endothelial dysfunction, combined with pro-inflammatory, pro-atherothrombotic and pro-coagulant (increased platelet aggregation and reduced fibrinolytic sensitivity) responses. Despite lower pro-inflammatory signals (increased insulin, cortisol and glucagon) and milder hypoglycemia, equivalent SNS drive resulted in an even greater acute pro-inflammatory and pro-atherothrombotic state (increased ICAM-1, VCAM-1, PAI-1, VEGF and Endothelin-1 responses) combined with greater endothelial dysfunction (reduced brachial artery flow mediated dilation) in the obese type 2

diabetes group compared to non-obese healthy controls. These present results may help provide a mechanistic pathophysiologic insight for the greater risk of severe cardiovascular adverse events and mortality following hypoglycemia that occurs in standard treated type 2 diabetes.

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N. J. performed studies, researched and analyzed data, contributed to writing, reviewing and edited the manuscript. M.M. helped perform studies. L. Y. perform studies, researched data and D. T. helped perform studies, researched data, and reviewed and edited the manuscript. S. D. devised the study, contributed to writing, reviewed and edited data and the manuscript. All are affiliated with the University of Maryland, Baltimore.

Stephen Davis is the guarantor of this study and, as such, had full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Highlights

- Study determined the pro-inflammatory and pro-atherothrombotic effects of hypoglycemia in Type 2 DM
- Sympathetic nervous system drive was equated during hypoglycemia in type 2 DM and healthy individuals
- ICAM-1, VCAM-1, PAI-1, P-selectin, Endothelin-1 and VEGF increased more during hypoglycemia in type 2 DM
- In-vivo vascular endothelial function was decreased by a greater extent during hypoglycemia in type 2 DM.

- Type 2 Diabetes Euglycemia
- Type 2 Diabetes Hypoglycemia
- ▼ Healthy Euglycemia
- ▽ Healthy Hypoglycemia

Figure 1.

Clamped glucose and insulin levels during hyperinsulinemic euglycemic and hypoglycemic clamps in healthy and type 2 diabetes individuals. Values are mean $\pm$ SE.

Figure 2.

Effects of hyperinsulinemic euglycemia (5 mmol/L) and hyperinsulinemic hypoglycemia (3.2 mmol/L and 2.9 mmol/L) on VCAM-1, ICAM-1, P-selectin and PAI-1 responses from baseline to end of clamps in overnight fasted type 2 diabetes and healthy individuals respectively. Values are mean $\pm$ SE. * p<0.01–0.009 significantly different from baseline, †p<0.01–0.005 significantly different from healthy individuals, ‡p<0.04–0.005 significantly different from euglycemia.

Figure 3.

Effects of hyperinsulinemic euglycemia (5 mmol/L) and hyperinsulinemic hypoglycemia (3.2 mmol/L and 2.9 mmol/L) on VEGF, Endothelin-1 and FMD. Responses from baseline to end of clamps in overnight fasted type 2 diabetes and healthy individuals. Values are mean±SE. *p<0.01–0.0001 significantly different from baseline, † p<0.05–0.004 significantly different from healthy individuals, ‡ p<0.02–0.004 significantly different from euglycemia, § p=0.05 significantly different from baseline.

BSL- baseline EC-end of clamp.

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Table 1

Healthy and Type 2 Diabetes Demographics

	Healthy	Type 2 Diabetes
Sex (male/female)	11M/5F	2M/9F
BMI	27±1.3	38±3 *
Age (yrs)	36±3	45±3.5
Race (CC,AA,As)	11CC/4AA/1As	5AA/6CC
HgA1c %	5±0.3	7.9±1 *
AST Units/L	27±1.2	27±2
ALT Units/L	23±2.5	27±4
Creatinine mg/dL	1±0.05	0.8±0.04
C-peptide ng/mL	2±0.3	1±0.3 *

CC- Caucasian AA- African American As- Asian

*
p<0.02–0.001 different from healthy

Baseline (BSL) and End of Clamp (EC) Levels of Neuroendocrine and Counterregulatory Hormones and NEFA Levels in overnight fasted healthy and type 2 diabetic individuals during hyperinsulinemic Day 1 Euglycemia and Day 2 Hypoglycemia

Table 2

SI units	Euglycemia Healthy		Euglycemia Type 2 diabetes		Hypoglycemia Healthy		Hypoglycemia Type 2 diabetes	
	BSL	EC	BSL	EC	BSL	EC	BSL	EC
Glucagon ng/L	64±5	43±3 *	75±6	60±7 ‡	56±4	124±16 *§	72±2 †	173±10 *‡§
Epinephrine pmol/L	190±20	189±20	229±62	277±91	179±19	4251±568 *§	161±33	4049±970 *§
Norepinephrine pmol/L	1083±80	1159±77	938±73	1003±59	1128±124	1957±134 *§	930±55	1907±155 *§
Cortisol nmol/L	344±31	292±30	416±69	376±75	330±32	714±42 *§	369±54	1003±64 *‡§
NEFA mmol/L	379±48	103±40 *	277±55	101±22 *	382±55	97±14 *	365±66	203±58 *‡§

NEFA= non esterified fatty acids BSL-baseline EC- end of clamp

* p= 0.01-0.001- EC different from BSL
‡ p= 0.02-0.001- ANOVA EC different in Type 2 Diabetes compared to healthy during hypoglycemia or euglycemia
‡ p= 0.003- ANOVA different from BSL hypoglycemia healthy
§ p=0.01- 0.001 – ANOVA EC different during hypoglycemia as compared to euglycemia

Table 3

Baseline (BSL) levels of pro-inflammatory, pro-atherothrombotic and pro-coagulation markers in overnight fasted healthy and type 2 diabetic individuals during hyperinsulinemic Day 1 Euglycemia and Day 2 Hypoglycemia

SI units	Euglycemia Healthy BSL	Euglycemia Type 2 diabetes BSL	Hypoglycemia Healthy BSL	Hypoglycemia Type 2 diabetes BSL
VCAM-1 ng/mL	838±40	693±59 *	772±45	536±37 *†
ICAM-1 ng/mL	91±8	124±17	89±7	111±12
P-Selectin pg/mL	83±10	64±6	57±6 *	52±10
E-Selectin ng/mL	22±2	23±4	18±2	19±3
PAI-1 ng/mL	18±3	27±14	14±2	19±3
Endothelin-1 fg/mL	883±67	812±109	925±109	741±90
VEGF pg/mL	27±8	166±58 *	22±6	232±87 †
tPA ng/mL	6.1±1	1.8±0.3 *	5.9±1	1.9±1 †
TNF α ng/mL	2.5±0.5	3.6±0.5	2.3±0.5	2.9±0.5

* p=0.007–0.02 ANOVA different from healthy euglycemia

† p=0.01–0.02 ANOVA different from healthy hypoglycemia

Table 4

Cardiovascular Responses during Hyperinsulinemic Euglycemic and Hypoglycemic clamps

	Basal	Final
Systolic Blood Pressure mmHg		
Healthy Day 1 eugly	110±3	110±3
Healthy Day 2 hypo	109± 3	116±4
Type 2 Diabetes Day 1 eugly	116±4	119±4
Type 2 Diabetes Day 2 hypo	116±6	126±7 [†]
Diastolic Blood Pressure mmHg		
Healthy Day 1 eugly	63±2	62±2
Healthy Day 2 hypo	65±1	60±2 [†]
Type 2 Diabetes Day 1 eugly	65±3	66±2
Type 2 Diabetes Day 2 hypo	67±3	68±4
Mean Arterial Blood Pressure mmHg		
Healthy Day 1 eugly	79±2	76±3
Healthy Day 2 hypo	80±2	80±2
Type 2 Diabetes Day 1 eugly	81±4	83±3
Type 2 Diabetes Day 2 hypo	83±4	84±8
Heart Rate Beats/minute		
Healthy Day 1 eugly	64±3	67±2
Healthy Day 2 hypo	65±3	74±2 ^{†‡}
Type 2 Diabetes Day 1 eugly	77±4	79±2
Type 2 Diabetes Day 2 hypo	77±4	79±5

[†] p<0.05 compared to baseline[‡] p<0.05 compared to final heart rate healthy eugly

Values are means ±SE

Eugly- Euglycemia

Hypo-Hypoglycemia